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(54) Title: COMPOSITIONS AND METHODS FOR THE DIMINUTION OR ELIMINATION OF VARIOUS CANCERS

(57) Abstract: The present invention relates to the treatment of cancer utilizing Jun Kinase antisense (JNK2AS) mRNA. Additionally, the present invention relates to the screening of reagents that may augment or mimic the effect of JNK2AS treatment for cancer.

COMPOSITIONS AND METHODS FOR THE DIMINUTION OR ELIMINATION OF VARIOUS CANCERS

BACKGROUND

Cancer is a worldwide problem. The American Cancer Society estimates that over one half million people will die from cancer in the United States alone in 1999. As such, finding novel compositions and methods for the treatment of cancer is of vital interest. Currently, the treatment of cancer falls into three general categories: chemotherapy, radiation therapy and surgery.

Each of these therapies have drawbacks. For example, surgical excision of cancerous growth is imprecise leaving cancer cells that can continue to spread. Chemotherapeutic agents can work in a number of ways. For example, a chemotherapeutic can work by interfering with cell cycle progression or by generating DNA strand breaks. If the cancer cell is not able to overcome the cell cycle blockage or cell injury caused by the therapeutic compound, the cell will often die via apoptotic mechanisms. However, chemotherapy is non-specific, targeting all proliferating cells, not only cancer cells. Therefore, administration of chemotherâpeutic agents must be carefully monitored. There is often a fine line between a therapeutically beneficial amount of drug and a toxic amount. Often times, the only level of drug that will be effective against the tumor is also toxic to the patient. Additionally, the surviving cancer cells often develop resistance to the chemotherapeutic agent used as well as other agents of similar chemical structure.

Radiation therapy works by generating DNA strand breaks in such an excessive amount that the cell cannot recover. Radiation therapy suffers from the same limitation as surgical removal of the tumor: it can be imprecise, not from a technical standpoint, however, but from the ability of the doctor to ensure that even the most minute trace of cancer has been located. Additionally, like chemotherapy, radiation therapy also has toxic side affects.

A typical solution to these problems is to combine the various treatments thereby lessening the dangers of side effects. For example, the surgical excision of large tumor masses is followed by chemotherapy and/or radiation therapy. However, as is evident by the high rate of cancer morbidity, even combination treatments are often ineffective.

What is needed are novel methods for the screening of new, more focused cancer fighting compounds as well as novel methods for the treatment of cancer.

SUMMARY OF THE INVENTION

The present invention relates to the use of antisense technology aimed to inhibit JNK2 expression for the screening of therapeutic compounds and for the treatment of various cancers. In this regard, the invention relates to the novel observation that inhibition of JNK2 expression by JNK2 antisense oligonucleotide (JNK2AS), inhibited tumor cell growth and induced an apoptotic response in a p53-deficient manner. The observation that JNK2AS oligonucleotide is capable of conferring an apoptotic response in certain cancer cells has not been made before now. It is important to distinguish the difference between cessation of cell growth and the induction of apoptosis from both physiological and therapeutic standpoints. The physiological basis for the two processes are ordinarily mutually exclusive. In other words, the cessation of growth is a separate phenomenon from the induction of apoptosis. From a therapeutic stand point, to eliminate cancer in a patient, it is necessary to kill the cancer cells, not just slow or inhibit their growth. Furthermore, the novel observation that inhibition of JNK2 function(s) in human tumor cells by JNK2AS induces apoptosis in p53-deficient cells allows for the therapeutic intervention of p53-deficient tumors and for the screening of compounds that mimic or augment JNK2AS activity. In this regard, the present invention relates, in part, to methods incorporating isolated and purified oligonucleotide sequences that encode Jun kinase 2 antisense oligonucleotides (JNK2AS). The present invention is not limited to any particular JNK2 antisense oligonucleotide. The present invention contemplates any oligonucleotide sequence that is capable of binding to JNK2 sense mRNA and inhibiting JNK2 expression and/or function(s). In one preferred embodiment, the antisense mRNA strand is at least 25% homologous to the JNK2 sense strand. In a more preferred embodiment, the antisense strand is at least 50% homologous to the JNK2 sense strand. In an even more preferred embodiment the antisense JNK2 strand is at least 75% homologous to the JNK2 sense strand. It is additionally contemplated that the antisense strand need not be composed of RNA. It is contemplated that the antisense JNK2 strand may be composed of DNA or of synthetic nucleotides. A JNK2AS strand composed of synthetic nucleotides may be, for example, more stable as a therapeutic.

The present invention also relates to peptides translated from the JNK2 antisense sequences described above. It is not intended that the translation of these peptides be limited to any particular translation system, as many translation systems are contemplated. Indeed, the peptides may be produced by any number of methods well know to those practiced in the art. For example, the peptides may be translated in cell-free systems, including but not limited to rabbit reticulocyte cell-free translation systems, *E. coli* cell-free translation systems and wheat germ cell-free translation systems. The peptides may also be translated in cell-based prokaryotic and eukaryotic translation systems. These cell-based translation systems are not limited to any particular cell type to be used for the production of the peptides. Furthermore, the cell-based translation systems are not limited to any particular expression vector. Additionally, the peptides may be synthesized using peptide a synthesizer.

The present invention contemplates the production of antibodies directed against the peptides synthesized from the oligonucleotides of the present invention. The present invention is not limited to any particular type of antibodies, providing the antibodies recognize at least a portion of the peptides translated from the oligonucleotides of the present invention. These antibodies may be produced by methods known to those practiced in the art. The antibodies may be polyclonal and/or monoclonal.

The present invention contemplates expression vectors wherein the vectors encode JNK2 antisense oligonucleotide (*i.e.* SEQ ID NOS:1 to 5). However, the present invention is not limited to any particular expression vector. The expression vector of the present invention may be any vector in which the sequence encoding the JNK2 antisense mRNA is operably linked. Methods for producing these operably linked expression vectors are well know to those practiced in the art. Additionally, the present invention contemplates expression vectors wherein the vectors that encode JNK2AS oligonucleotide comprise promoter regions that are tissue or cell type specific. Furthermore, the present invention contemplates cells comprising expression vectors wherein the cells express JNK2 antisense oligonucleotide. The present invention is not limited to any particular cell type. Indeed, both prokaryotic and eukaryotic cells are contemplated by the present invention. Also, the cells may be transfected *in situ* or *in vitro*.

The present invention contemplates the oligonucleotides of SEQ ID NO:1 to 5 linked to a peptide. The present invention is not limited by the method in which the oligonucleotide sequence is linked to the peptide. However, in one embodiment, the

linkage is covalent. The present invention is likewise not limited by the peptide to which the oligonucleotide of SEQ ID NO: 1 to 5 is linked. However, in one embodiment, the peptide is a target for a cell surface receptor. In another embodiment the peptide is a target for a cell surface receptor which internalizes upon binding to its target peptide.

The present invention contemplates methods for the production of the sequences of SEQ ID NO: 1 to 5, by any means known in the art suitable for such production. Thus, the present invention is not limited by the methods employed for the production of SEQ ID NO: 1 to 5. The present invention contemplates producing the sequences of SEQ ID NO:1 to 5 by, for example, an oligonucleotide synthesizer, prokaryotic and eukaryotic transcription, polymerase chain reaction (PCR) or any other suitable amplification method, cell-free transcription and by cleavage and ligation of known or random sequences of oligonucleotide where the sequences may be as small as one nucleotide.

The present invention also contemplates methods for the screening for compounds that mimic or augment the activity of JNK2AS oligonucleotide in cells. Although the present invention is not dependent on the mechanism by which the compound mimics or augments JNK2AS activity, the compound may bind JNK mRNA sense strands and may affect the JNK2 protein expression and, therefore, interfere with its function(s), or the compound may interact with other cellular components related or unrelated to the JNK pathway resulting in the modulation of the JNK2 expression and/or function(s). The present invention is not limited to the method used for the screening of compounds that mimic or augment the JNK2AS activity. In one embodiment the present invention provides methods involving 1) providing, i) one or more compounds, ii) p53-deficient cells, a portion of which are transfected with JNK2AS oligonucleotide to produce transfected and non-transfected cells, 2) contacting the non-transfected cells with the compound(s) to produce treated, non-transfected cells, 3) measuring the effect of the compound and measuring the effect of JNK2AS oligonucleotide transfection on the transfected cells, and 4) comparing the effect of treated non-transfected cells with transfected cells.

The present invention relates to the use of JNK2 antisense (JNK2AS) for the purpose of inhibiting growth and inducing cell death (apoptosis) in cancer cells. This invention particularly relates to the reduction or cessation of cancer in tumors comprising p53-deficient cells. In this regard, the present invention contemplates methods for the

treatment of cancer. The present invention is not limited to any particular method. However, in one embodiment, the present invention provides methods involving 1) providing, i) a patient diagnosed with a cancer suspected of responding to JNK2AS treatment, ii) one or more expression vectors comprising a JNK2AS oligonucleotide having a sequence of SEQ ID NO:2 (or equivalent) or a portion thereof, and a cell-targeting mechanism, 2) administering the expression vector or vectors and the targeting mechanism to the patient, and 3) monitoring the patient's disease. The present invention is not limited to any particular targeting mechanism. For example, in another embodiment, the targeting method is a peptide covalently linked to the expression vector that is a target peptide specific for a receptor on the cancer cells. In yet another embodiment, the administration of the vector(s) and targeting mechanism may be by a single dose or multiple doses. Additionally, the administration may be continuous or non-continuous. Furthermore, the treatment may include the administration to the patient of a compound or other agent for the purpose of rendering a p53-nondeficient cell p53-deficient. The compound or agent is not limited by its nature or composition. For example, the compound or agent may be p53AS oligonucleotide or a compound known or suspected of rendering p53-nondeficient cells p53-deficient. It is also contemplated that all compounds, agents or antisense oligonucleotide may be combined with a pharmacologically acceptable carrier.

The present invention contemplates the *in situ* transfection of cells in a host or patient by methods known to those practiced in the art. For example, JNK2AS oligonucleotide maybe injected directly into the tumor. In another example, it is contemplated that cells may be transfected by using viral vectors. In one embodiment, vaccinia virus vectors are used (Lattime *et al.*,Semin Onocol., 23:88-100 [1996]). In another embodiment, recombinant adenovirus and herpes simplex virus vectors (Yang, Crit. Rev. Biotechnol., 12:335-356 [1992]) are also contemplated for the *in situ* transfection of operably linked JNK2AS encoding regions. In yet another embodiment, microparticle injection (Johnson and Tang, *supra*), particle bombardment (Yang, *supra*) and gene gun transfection (Johnson and Tang, Meth, Cell Biol., 43 PtA:353-365 [1994]) are also contemplated for the *in situ* transfection of operably linked JNK2AS encoding regions. In yet another embodiment, direct injection of expression vectors into tissue is contemplated for the *in situ* transfection of operably linked JNK2AS encoding regions. In this regard, it is contemplated that the expression vector is linked to a targeting

molecule that is recognized by a tissue-specific receptor. In a preferred embodiment, the tissue-specific receptor may be internalized upon ligation with the targeting molecule.

DESCRIPTION OF FIGURES

Figure 1 shows that MCF-7 and RKO cells display high efficiency uptake of oligonucleotides delivered via transfection with Lipofectin reagent. Cells were treated with 0.3 μM 3'FITC-labeled control oligonucleotide. Twenty-four hours later, they were fixed and examined for fluorescence using a confocal microscope. Images were taken at 800x magnification. (Left panel) confocal fluorescent images of transfected cells. (Right panel) confocal transparent images of the same fields. Virtually all cells show uptake of the oligonucleotides, irrespective of p53 status.

Figure 2 shows that JNKAS treatment effectively inhibits *JNK* expression in MCF-7 and RKO cells regardless of p53 status. (A) Total RNA was extracted from cells 24 h following treatment with 0.3 μM of either JNK1AS, JNK2AS, or control oligonucleotides (JNKScr). RNA samples were analyzed by Northern analysis using cDNA probes specific for *JNK1* and *JNK2* mRNAs. (B) Whole-cell lysates were examined by Western analysis for JNK expression 24 h following treatment with JNKAS oligonucleotides. (C) Total Jun kinase activity was determined 30 min following exposure to UVC (40 J/m^2) by an *in vitro* kinase assay using GST-cJun(1-222) as a substrate. Twenty four hours prior to UVC treatment, cells were transfected with combinations of JNK1AS + JNK2AS (0.15 μM each) or JNK1Scr + JNK2Scr (0.15 μM each) labeled JNKAS and JNKScr, respectively.

Figure 3 shows the effect of JNKAS treatment on viability of p53-proficient and p53-deficient MCF-7 and RKO cells. Cells were seeded in 96-well cluster plates and treated with 0.3 μM oligonucleotides in Lipofectin reagent ("JNKScr" designates treatment with 0.15 mM of each JNK1Scr and JNK2Scr). Cell viability was assessed at designated times by measuring MTS tetrazolium dye in a colorimetric assay as described in the Examples.

Figure 4 shows the morphological appearance of JNK2AS-treated MCF-7 and RKO cells with normal (neo) or deficient (E6) p53 function. Cells treated with 0.3 μM of the indicated oligonucleotides were examined by optical microscopy 24 h after lipofection. Cultures that were either subjected to mock lipofection or to treatment with scrambled oligonucleotides (same as described in Figure 3) had similar morphology to

those seen in untreated control cultures. The density of JNKAS-treated E6-expressing MCF-7 and RKO cells is lower than that of the neo-expressing counterparts. JNK2AS-treated p53-deficient (E6-expressing) cells exhibit membrane blebbing, and an increase in number of rounded and/or detached cells.

Figure 5 shows biochemical evidence of DNA degradation in p53-deficient cells treated with JNKAS. Apoptosis of p53-proficient (neo) and p53-deficient (E6-expressing) MCF-7 and RKO cells was assessed 24 h after treatment with 0.3 μM JNKAS or control oligonucleotides using an ELISA assay that measures nucleosomal degradation. JNKScr depicts results for treatment with equal amounts (0.15 μM) of JNK1Scr and JNK2Scr. Results are expressed as relative absorption at 405 nm.

Figure 6 shows FACS analysis of mock-transfected and JNKAS-treated RKO cells. RKO neo and RKO E6-expressing cells were treated with either JNK1AS, JNK2AS, or a combination of control scrambled oligonucleotides (JNKScr). They were harvested 24 h following the lipofection, and 2 x 10^6 cells were subjected to DNA content analysis by FACS. The arrow indicates the sub-G1 peak indicating apoptotic cells. The percentage of total cells contained in the sub-G1 peak of JNK2AS and JNK1+2AS-treated RKO E6 cell cultures are indicated on the figure. For all other treatments of E6-expressing RKO and RKO neo cells the sub-G1 fraction was 1.5 % or less.

Figure 7 shows that the inhibition of *JNK* expression in HCT116 cells results in growth suppression in a p53-dependent manner. Viable cell mass was measured at 24, 48 (shown here) and 72 h following treatment with JNKAS and control oligonucleotides as described in the Examples section. Growth inhibition following JNK2AS treatment is p53-dependent: "*" designates statistically significant difference between number of viable cells in JNK2AS-treated cultures of parental HCT116 cells and HCT116 $p53^{-/-}$ cells (Student's test, $p < 0.003$).

Figure 8 shows JNK2AS treatment results in induction of $p21^{Cip1/Waf1}$ expression in cells with normal p53 function (neo-transfected MCF-7 and RKO, and HCT166 cells), but not in p53-deficient cells (E6-expressing MCF-7 and RKO, and HCT116 $p53^{-/-}$ cells). (A) Total RNA was extracted from cells 24 h following treatment with 0.3 μM of either antisense (AS) or control oligonucleotides (Scr). RNA samples were analyzed by Northern analysis using an oligonucleotide probe specific for human $p21^{Cip1/Waf1}$. Following analysis of $p21^{Cip1/Waf1}$ expression, Northern blots were stripped and re-probed

with oligonucleotide complementary to 18S rRNA probe to verify equal loading and transfer of RNA samples. (B) $p21^{Cip1/Waf1}$ expression was examined by Western blot analyses 24 h following treatment with 0.3 mM of JNK2AS or JNKScr oligonucleotides (as described in Figure 3).

Figure 9 shows that HCT116 human colorectal carcinoma cells lacking $p21^{Cip1/Waf1}$ expression display enhanced sensitivity to JNK2AS treatment. Parental HCT116 cells with normal $p21^{Cip1/Waf1}$ expression (p21+/+), and derivative lines in which one (p21+/-) or both (p21-/-) $p21^{Cip1/Waf1}$ alleles have been disrupted through homologous recombination, were examined by FACS analysis for apoptotic cells 24 h following treatment with JNK1AS or JNK2AS oligonucleotides.

Figure 10 shows PC3 xenograft arrest by antisense JNK and cisplatin. (A) the average tumor volume for each treatment is plotted as a function of time. The symbols used in this Figure are as follows: control phosphorothioate, ; antisense JNK (JNK1AS^{ISIS12539} and JNK2ASISIS^{12560}), ▲; untreated, ●; combination antisense JNK (JNK1AS^{ISIS12539} and JNK2ASISIS^{12560}) plus weekly doses of cisplatin, ▼; or cisplatin alone, ◀. (B) Tumor regrowth following cessation of treatment ("END" arrow). For antisense JNK treated group, ▲; no intact tumors remained after d=38 (see tumor regression, Figure 2).

Figure 11 shows regression of established tumors by systematic antisense JNK and cisplatin treatment. (A) Percent of animals in each treatment group (Figure 1) that developed distal ulcers as a function of time (arrows indicate treatment period). (B) Time course of growth of average tumor ulcer diameter. In cases where ulcers were not spherical, perpendicular diameters were averaged.

Figure 12 shows that JNK activity and tumor viability is reduced in antisense JNK-treated tumors. (A) JNK activity was measured for equal amounts of tumor extract based on protein determinations and confirmed by β-actin measurements in most cases. JNK activity is high in PC3 cells and control ("Cont") oligonucleotide-treated tumors but low in quiescent PC3 cells, in all antisense JNK treated tumors as it is for most tissue, here kidney (lane 10, determined in a separate experiment in parallel with a replicate control oligonucleotide-treated tumor sample, lane 9). (B) Histology of pending ulceration (hematoxylin-eosin, 10X; bar = 3,000 μm).

Figure 13 shows inhibition of tumorigenesis by dominant negative c-Jun(S63A, S73A).

Figure 14 shows the depletion of JNK results in growth inhibition of T98G cells. (A) and (B), assessment of the viable cell mass by MTS tetraxolium dye conversion at 24 h (A) and 120 h (B) following treatment with oligonucleotides. (C), effect of JNKAS treatment on cell number and portion of attached cells.

Figure 15 shows growth inhibition by JNK1AS and JNK2AS is dose-dependent.

Figure 16 shows the morphology of JNKAS-treated cells (magnification at x 400).

Figure 17 shows apoptosis analysis following treatment with JNKAS. (A), detection of apoptotic bodies in DAPI-stained cells at x 400 magnification. (B), detection of apoptotic cell fractions using APO-BRDU kit (PharMingen). Apoptosis-positive cells are located in the upper right hand corner of the histograms: 22% in positive control sample and less than 1% in all other samples.

Figure 18 shows the cell cycle analysis following treatment with JNKAS. (A), flow cytometric analysis of PI-stained cells 24 h postlipofection. (B), detection of s-phase cells using BrdUrd pulse incorporation. (C), shows graphic representation of the cell cycle analysis of Figure 18A.

Figure 19 shows (A), the inhibition of DNA synthesis after treatment with JNKAS and (B), the recovery of DNA synthesis following treatment with JNK1AS but not with JNK2AS.

Figure 20 shows the induction of $p21^{cip/waf1}$ expression in JNK2AS-treated cells and associated inhibition of cyclin-dependent kinase activities. (A) shows a Northern analysis of $p21^{cip/waf1}$ gene expression 24 h pot-treatment with 0.4 μM of either JNK1AS, JNK2AS, JNK1Scr or JNK2Scr oligonucleotides. (B) shows a Western blot analysis for expression of cell cycle regulatory proteins in JNK1AS- and JNK1AS-treated cultures. (C) shows inhibition of Cdk2/Cdc2 kinase activities in JNK1AS- and JNK2AS-treated cultures.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

"Nucleic acid sequence," "nucleotide sequence" and "polynucleotide sequence" as used herein refer to an oligonucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or

double-stranded, and represent the sense or antisense strand.

As used herein, the terms "oligonucleotides" and "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a probe or amplimer.

The term "nucleotide sequence of interest" refers to any nucleotide sequence, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. Such nucleotide sequences include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and of non-coding regulatory sequences do not encode an mRNA or protein product (*e.g.*, promoter sequence, enhancer sequence, polyadenylation sequence, termination sequence, *etc.*).

"Amino acid sequence," "polypeptide sequence," "peptide sequence," and "peptide" are used interchangeably herein to refer to a sequence of amino acids.

A "variant" of a nucleotide sequence is defined as a nucleotide sequence which differs from the referenced, parent or wild type nucleotide sequence (*e.g.*, by having one or more deletions, insertions, or substitutions that may be detected using hybridization assays or using DNA sequencing). Included within this definition is the detection of alterations to the genomic sequence of the nucleotide sequence. For example, hybridization assays may be used to detect alterations in: (1) the pattern of restriction enzyme fragments capable of hybridizing to a genomic sequence of the first nucleotide sequence (*i.e.*, RFLP analysis), (2) the inability of a selected portion of the first nucleotide sequence to hybridize to a sample of genomic DNA which contains the first nucleotide sequence (*e.g.*, using allele-specific oligonucleotide probes), (3) improper or unexpected hybridization, such as hybridization to a locus other than the normal chromosomal locus for the first nucleotide sequence (*e.g.*, using fluorescent *in situ* hybridization (FISH) to metaphase chromosomes spreads, etc.). One example of a variant is a mutated wild type sequence.

The term "portion" when used in reference to a nucleotide sequence refers to fragments of that nucleotide sequence. The fragments may range in size from 5 nucleotide residues to the entire nucleotide sequence minus one nucleic acid residue.

An oligonucleotide sequence which is a "homolog" of a first nucleotide sequence is defined herein as an oligonucleotide sequence which exhibits greater than or equal to

50% identity, and more preferably greater than or equal to 70% identity, to the first nucleotide sequence when sequences having a length of 10 bp or larger are compared.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is referred to as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of another mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects that transcription proceeds in a 5' to 3' direction along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another.

The term "expression vector" or "expression cassette" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The terms also refer to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "transfection" as used herein refers to the introduction of foreign DNA into cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (*i.e.*, particle bombardment) and the like.

As used herein, the terms "complementary" or "complementarity" are used in reference to "polynucleotides" and "oligonucleotides" (which are interchangeable terms that refer to a sequence of nucleotides) related by the base-pairing rules. For example, the sequence "5'-CAGT-3'," is complementary to the sequence "5'-ACTG-3'." Complementarity can be "partial" or "total." "Partial" complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules. "Total" or "complete" complementarity between nucleic acids is where each and every nucleic acid base is matched with another base under the base pairing rules. The degree of complementarity between nucleic acid strands may have significant effects on the efficiency and strength of hybridization between nucleic acid strands. This may be of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The terms "homology" and "homologous" as used herein in reference to nucleotide sequences refer to a degree of complementarity with other nucleotide sequences. There may be partial homology or complete homology (*i.e.*, identity). A nucleotide sequence which is partially complementary, *i.e.*, "substantially homologous," to a nucleic acid sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid sequence. The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the

hybridization) of a completely homologous sequence to a target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

Low stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $NaH_2PO_4 \cdot H_2O$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1 % SDS, 5X Denhardt's reagent (50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)) and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 2.0X SSPE, 0.1% SDS at room temperature when a probe of about 100 to about 1000 nucleotides in length is employed.

It is well known in the art that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, *etc.*) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol), as well as components of the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, conditions which promote hybridization under conditions of high stringency (*e.g.*, increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, *etc.*) are well known in the art. High stringency conditions, when used in reference to nucleic acid hybridization, comprise conditions equivalent to binding or hybridization at 68°C in a solution consisting of 5X SSPE, 1 % SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE and 0.1 % SDS at 68°C when a probe of about 100 to about 1000 nucleotides in length is employed.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize to either or both strands of the double-stranded nucleic acid sequence under

conditions of low stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe which can hybridize (*i.e.*, it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids using any process by which a strand of nucleic acid joins with a complementary strand through base pairing to form a hybridization complex. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids.

As used herein the term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bounds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (*e.g.*, C_0t or R_0t analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized to a solid support (*e.g.*, a nylon membrane or a nitrocellulose filter as employed in Southern and Northern blotting, dot blotting or a glass slide as employed in *in situ* hybridization, including FISH (fluorescent *in situ* hybridization)).

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.*, Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m.

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic

solvents, under which nucleic acid hybridizations are conducted. "Stringency" typically occurs in a range from about T_m°C to about 20°C to 25°C below T_m. As will be understood by those of skill in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under "stringent conditions" the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, or portions thereof, will hybridize to its exact complement and closely related sequences.

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

The term "heterologous nucleic acid sequence" or "heterologous DNA" are used interchangeably to refer to a nucleotide sequence which is ligated to a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of a target sequence of interest. In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

"Amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art (Dieffenbach and Dveksler, *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY [1995]). As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods of U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, all of which are hereby incorporated by reference, which describe a method

for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. The length of the amplified segment of the desired target sequence is determined by the relative positions of two oligonucleotide primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ^{32}P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

The terms "reverse transcription polymerase chain reaction" and "RT-PCR" refer to a method for reverse transcription of an RNA sequence to generate a mixture of cDNA sequences, followed by increasing the concentration of a desired segment of the transcribed cDNA sequences in the mixture without cloning or purification. Typically, RNA is reverse transcribed using a single primer (*e.g.*, an oligo-dT primer) prior to PCR amplification of the desired segment of the transcribed DNA using two primers.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and of an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the

primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that it is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double- or single-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "an oligonucleotide having a nucleotide sequence encoding a gene" means a nucleic acid sequence comprising the coding region of a gene, *i.e.* the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers, promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

Transcriptional control signals in eukaryotes comprise "enhancer" elements. Enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis *et al.*, Science 236:1237 [1987]). Enhancer elements have been isolated from a variety of eukaryotic sources including genes in plant, yeast, insect and mammalian cells and viruses. The selection of a

particular enhancer depends on what cell type is to be used to express the protein of interest.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, pp. 16.7-16.8 [1989]). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene.

The term "promoter," "promoter element," or "promoter sequence" as used herein, refers to a DNA sequence which when placed at the 5' end of (*i.e.*, precedes) an oligonucleotide sequence is capable of controlling the transcription of the oligonucleotide sequence into mRNA. A promoter is typically located 5' (*i.e.*, upstream) of an oligonucleotide sequence whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and for initiation of transcription.

The term "promoter activity" when made in reference to a nucleic acid sequence refers to the ability of the nucleic acid sequence to initiate transcription of an oligonucleotide sequence into mRNA.

The term "tissue specific" as it applies to a promoter refers to a promoter that is capable of directing selective expression of an oligonucleotide sequence to a specific type of tissue in the relative absence of expression of the same oligonucleotide in a different type of tissue. Tissue specificity of a promoter may be evaluated by, for example,

operably linking a reporter gene to the promoter sequence to generate a reporter construct, introducing the reporter construct into the genome of an animal such that the reporter construct is integrated into every tissue of the resulting transgenic animal, and detecting the expression of the reporter gene (*e.g.*, detecting mRNA, protein, or the activity of a protein encoded by the reporter gene) in different tissues of the transgenic animal. Selectivity need not be absolute. The detection of a greater level of expression of the reporter gene in one or more tissues relative to the level of expression of the reporter gene in other tissues shows that the promoter is specific for the tissues in which greater levels of expression are detected.

The term "cell type specific" as applied to a promoter refers to a promoter which is capable of directing selective expression of an oligonucleotide sequence in a specific type of cell in the relative absence of expression of the same oligonucleotide sequence in a different type of cell within the same tissue. The term "cell type specific" when applied to a promoter also means a promoter capable of promoting selective expression of an oligonucleotide in a region within a single tissue. Again, selectivity need not be absolute. Cell type specificity of a promoter may be assessed using methods well known in the art, *e.g.*, immunohistochemical staining as described herein. Briefly, tissue sections are embedded in paraffin, and paraffin sections are reacted with a primary antibody which is specific for the polypeptide product encoded by the oligonucleotide sequence whose expression is controlled by the promoter. A labeled (*e.g.*, peroxidase conjugated) secondary antibody which is specific for the primary antibody is allowed to bind to the sectioned tissue and specific binding detected (*e.g.*, with avidin/biotin) by microscopy.

The terms "selective expression," "selectively express," and grammatical equivalents thereof refer to a comparison of relative levels of expression in two or more regions of interest. For example, "selective expression" when used in connection with tissues refers to a substantially greater level of expression of a gene of interest in a particular tissue, or to a substantially greater number of cells which express the gene within that tissue, as compared, respectively, to the level of expression of, and the number of cells expressing, the same gene in another tissue (*i.e.*, selectivity need not be absolute). Selective expression does not require, although it may include, expression of a gene of interest in a particular tissue and a total absence of expression of the same gene in another tissue. Similarly, "selective expression" as used herein in reference to cell

types refers to a substantially greater level of expression of, or a substantially greater number of cells which express, a gene of interest in a particular cell type, when compared, respectively, to the expression levels of the gene and to the number of cells expressing the gene in another cell type.

The term "contiguous" when used in reference to two or more nucleotide sequences means the nucleotide sequences are ligated in tandem either in the absence of intervening sequences, or in the presence of intervening sequences which do not comprise one or more control elements.

The term "transfection" or "transfected" refers to the introduction of foreign DNA or RNA into a cell.

As used herein, the terms "nucleic acid molecule encoding," "nucleotide encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the term "antisense" is used in reference to RNA sequences which are complementary to a specific RNA sequence (*e.g.*, mRNA). Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a coding strand. Once introduced into a cell, this transcribed strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated or production of gene products may be reduced or inhibited. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand.

"JNK2AS" refers to antisense mRNA strands that are complementary to JNK2 mRNA sense strands. The complementation need not be complete or absolute. The binding of the antisense strand to the sense strand may inhibit the translation of the sense strand into a peptide by competitive or noncompetitive inhibition.

The term "Southern blot" refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size, followed by transfer and immobilization of

the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (Sambrook *et al.*, *supra*, at pp 9.31-9.58).

The term "Northern blot" as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (Sambrook *et al.*, *supra*, at pp 7.39-7.52).

The term "reverse Northern blot" as used herein refers to the analysis of DNA by electrophoresis of DNA on agarose gels to fractionate the DNA on the basis of size followed by transfer of the fractionated DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled oligo-ribonucleotide probe or RNA probe to detect DNA species complementary to the oligo-ribonucleotide probe used.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" refers to a nucleic acid sequence that is separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is nucleic acid present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA which are found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs which encode a multitude of proteins. However, isolated nucleic acid encoding a polypeptide of interest includes, by way of example, such nucleic acid in cells ordinarily expressing the polypeptide of interest where the nucleic acid is in a chromosomal or extrachromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic

acid sequence than that found in nature. The isolated nucleic acid or oligonucleotide may be present in single-stranded or double-stranded form. Isolated nucleic acid can be readily identified (if desired) by a variety of techniques (*e.g.*, hybridization, dot blotting, etc.). When an isolated nucleic acid or oligonucleotide is to be utilized to express a protein, the oligonucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide may be double-stranded).

As used herein, the term "purified" or "to purify" refers to the removal of one or more (undesired) components from a sample. For example, where recombinant polypeptides are expressed in bacterial host cells, the polypeptides are purified by the removal of host cell proteins thereby increasing the percent of recombinant polypeptides in the sample.

As used herein, the term "substantially purified" refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free and more preferably 90% free from other components with which they are naturally associated. An "isolated polynucleotide" is, therefore, a substantially purified polynucleotide.

As used herein the term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

As used herein, the term "structural gene" or "structural nucleotide sequence" refers to a DNA sequence coding for RNA or a protein which does not control the expression of other genes. In contrast, a "regulatory gene" or "regulatory sequence" is a structural gene which encodes products (*e.g.*, transcription factors) which control the expression of other genes.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements include splicing signals, polyadenylation signals, termination signals, *etc.*

As used herein, the term "peptide transcription factor binding site" or "transcription factor binding site" refers to a nucleotide sequence which binds protein transcription factors and, thereby, controls some aspect of the expression of nucleic acid sequences. For example, Sp-1 and AP1 (activator protein 1) binding sites are examples of peptide transcription factor binding sites.

As used herein, the term "gene" means the deoxyribonucleotide sequences comprising the coding region of a structural gene. A "gene" may also include non-translated sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into heterogenous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3' flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "oncogene" refers to a gene which is capable of transforming a normal cell to a cancer cell. An oncogene may be a viral oncogene or a cellular oncogene. A "viral oncogene" may be an early gene of a DNA virus (*e.g.*, polyomavirus, papillomavirus, T-cell leukemia virus), or a cellular proto-oncogene incorporated into the

genome of a transducing retroviruses such that the cellular proto-oncogene (*e.g.*, c-src) is activated into an oncogene (*e.g.*, v-src). In contrast to a viral oncogene, a "cellular oncogene" is a mutated cellular gene formed *in situ* in the chromosome of a cell rather than introduced into the cell by a DNA virus or a transducing virus.

The term "cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described (Pitot, *in Fundamentals of Oncology*, Marcel Dekker (Ed.), New York pp 15-28 [1978]). The features of early, intermediate and advanced stages of neoplastic progression have been described using microscopy. Cancer cells at each of the three stages of neoplastic progression generally have abnormal karyotypes, including translocations, inversion, deletions, isochromosomes, monosomies, and extra chromosomes. A cell in the early stages of malignant progression is referred to as "hyperplastic cell" and is characterized by dividing without control and/or at a greater rate than a normal cell of the same cell type in the same tissue. Proliferation may be slow or rapid but continues unabated. A cell in the intermediate stages of neoplastic progression is referred to as a "dysplastic cell." A dysplastic cell resembles an immature epithelial cell, is generally spatially disorganized within the tissue and loses its specialized structures and functions. During the intermediate stages of neoplastic progression, an increasing percentage of the epithelium becomes composed of dysplastic cells. "Hyperplastic" and "dysplastic" cells are referred to as "pre-neoplastic" cells. In the advanced stages of neoplastic progression a dysplastic cell become a "neoplastic" cell. Neoplastic cells are typically invasive, *i.e.*, they either invade adjacent tissues, or are shed from the primary site and circulate through the blood and lymph to other locations in the body where they initiate secondary cancers. The term "cancer" or "neoplasia" refers to a plurality of cancer cells.

A "non-human animal" refers to any animal which is not a human and includes vertebrates such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc. Preferred non-human animals are selected from the order Rodentia.

A "transgenic animal" as used herein refers to an animal that includes a transgene which is inserted into a cell and which becomes integrated into the genome either of somatic and/or germ line cells of the. A "transgene" means a DNA sequence which is partly or entirely heterologous (*i.e.*, not present in nature) to the animal in which it is found, or which is homologous to an endogenous sequence (*i.e.*, a sequence that is found

in the animal in nature) and is inserted into the animal's genome at a location which differs from that of the naturally occurring sequence. Transgenic animals which include one or more transgenes are within the scope of this invention.

The term "compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Compounds comprise both known and potential therapeutic compounds. A compound can be determined to be therapeutic by testing using the testing methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

A compound is said to be "in a form suitable for administration such that the compound is bio-available in the blood of the animal" when the compound may be administered to an animal by any desired route (*e.g.*, oral, intravenous, subcutaneous, intrathecal, intraperitoneal, intramuscular, etc.) and the compound or its active metabolites appears in the blood of the animal in an active form.

The terms "anti-neoplastic" and "anti-cancer" refer to a compound or procedure which arrests or retards the rate of neoplastic progression (*e.g.*, cancer cell growth or proliferation) and/or induced cell death, *e.g.* via apoptotic mechanisms. The term also refers to a compound which reduces the number of cancer cells in the absence of a change in the rate of neoplastic progression. Anti-neoplastic compounds may be naturally occurring as well as man-made.

As used herein "agonist" refers to molecules or compounds which mimic or augment the action of a "native" or "natural" compound. Agonists may be homologous to these natural compounds in respect to conformation, charge or other characteristics

As used herein "antagonist" refers to molecules or compounds which inhibit the action of a "native" or "natural" compound. Antagonists may or may not be homologous to these natural compounds in respect to conformation, charge or other characteristics.

"Patient" shall be defined as a human or other animal, such as a guinea pig or mouse and the like, that may be in need of alleviation or amelioration from a recognized medical condition.

"Apoptosis" and related terms such as "apoptotic" refers to a genetically controlled form of cell death characterized by, usually, one or more of the following: condensation of the nuclei, cell membrane blebbing, cleavage of the DNA into

nucleosomal sized fragments and the breakup of the cell into "apoptotic bodies".

"Proliferation" refers to the ability of cells to divide into two cells repeatedly thereby resulting in a total increase of cells in the population. Said population may be in an organism or in a culture apparatus.

A "cell-targeting mechanism" refers to a process, procedure or reagent that allows a compound or reagent (*e.g.* an antisense mRNA strand) to locate to a cell or cells in an organism. The targeting need not be absolute. The targeting need not be specific for a particular type of cell. A example of a targeting mechanism is a peptide (that is bound to, for example, an antisense mRNA strand) that may be recognized by a cell surface receptor so that, if in proximity to the cell surface receptor, the peptide will be bound by the cell surface receptor, and, thereby, be localized or targeted to the cell displaying the cell surface receptor, thereby also targeting the bound antisense mRNA to the cell.

A "transformed cell" is a cell or cell line that has acquired the ability to grow in cell culture for many multiple generations, the ability to grow in soft agar and the ability to not have cell growth inhibited by cell-to-cell contact. In this regard, transformation refers to the introduction of foreign genetic material into a cell or organism. Transformation may be accomplished by any method known which permits the successful introduction of nucleic acids into cells and which results in the expression of the introduced nucleic acid. "Transformation" includes but is not limited to such methods as transfection, microinjection, electroporation, and lipofection (liposome-mediated gene transfer). Transformation may be accomplished through use of any expression vector. For example, the use of baculovirus to introduce foreign nucleic acid into insect cells is contemplated. The term "transformation" also includes methods such as P-element mediated germline transformation of whole insects. Additionally, transformation refers to cells that have been transformed naturally, usually through genetic mutation.

"Wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and/or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these

are typically identified since they have altered characteristics when compared to the wild-type gene or gene product.

"Deficient" shall refer to a gene or gene product that has an altered characteristic or charateristics when compared to the wild-type gene. The altered characteristic(s) is (are) typically manifested by reduced or absent functionality. The gene or gene product may be physically altered or absent when compared to the wild-type. Alternately, the gene or gene product may not be altered but may show reduced or absent functionality due to changes in other aspects of the cell's or organism's physiology.

"p53-deficient" and "p53 null" shall refer to cells or organisms with reduced or absent p53 functionality. In one embodiment, the p53 gene is nonfunctional or absent.

"p53-mutant" shall refer to cells or organiams with p53 nucleotide and amino acid sequences the differ from the wild-type. In one embodiment, p53-mutant cells and organisms have less p53 functionality than wild-type cells or organisms. In another embodiment, p53-mutant cells and organisms have no p53 functionality.

GENERAL DESCRIPTION OF THE INVENTION

The present invention generally relates to the use of JNK2 antisense (JNK2AS) mRNA for the purpose of inhibiting growth and inducing cell death (apoptosis) in cancer cells. This invention particularly relates to the reduction or cessation of cancer in tumors comprising p53-deficient cells. The present invention also relates to drug screens for compounds that mimic or augment JNK2AS activity. Although, the present invention is not limited to any particular mechanism, the following discussion relates to the role of JNK function in cells. The c-Jun N-terminal Kinase (JNK) signal transduction pathway, culminating in the phosphorylation of one or more JNK proteins, is known to play an important role in coordinating the cellular response to stress (Ip and Davis, Curr. Op. Cell Biol., 10:205-19 [1998]; Minden and Karin, Biochim. Biophys. Acta 1333:F85-104 [1997]). The JNK family includes three genes, *JNK1*, *JNK2*, and *JNK3*, each of which can produce 46-kDa and 54-kDa isoforms (Ip and Davis, *supra*). *JNK1* and *JNK2* are ubiquitously expressed, while *JNK3* is largely restricted to brain, heart and testis (Ip and Davis, *supra*); Kumagae *et al.*, Brain Res. Mol. Brain Res., 67:10-7 [1990]; and Mohit *et al.*, Neuron 14:67-78 [1995]). Although some differences in the substrate specificities and activities of various JNK isoforms have been reported (Bost *et al.*, Mol. Cell. Biol., 19:1938-1949 [1999a]; and Gupta *et al.*, EMBO J. 15:2760-2770 [1996]), their

functional distinctions remain unclear. Among the major targets of JNK phosphorylation are the transcription factors c-Jun (Dérijard *et al.*, Cell 76:1025-37 [1994]) and tumor suppressor protein p53 (Milne *et al.*, J. Biol. Chem., 270:5511-5518 [1995]; and Hu *et al.*, Oncogene 15:2277-87 [1997]). Numerous studies have implicated both JNK activation and c-Jun phosphorylation in the induction of apoptosis following stress (Karin *et al.*,Curr. Opin. Cell Biol., 9:240-6 [1997]). However, an opposing function of the JNK pathway has also been suggested by other studies including two recent reports, which demonstrate that JNK-mediated phosphorylation of c-Jun confers protection to cells exposed to UVC irradiation or TNF-α (Behrens *et al.*, Nat. Genet., 21:326-9 [1999]; Wisdom *et al.*, EMBO J. 18:188-97 [1999]).

In addition to playing a role during stress, there is also evidence to support a role for the JNK pathway in regulating cell growth. While the basal activity of JNK is generally low in cells maintained under normal growth conditions, JNK can be activated by growth factors such as EGF and PDGF via mechanisms that appear to rely on PI-3 kinase (Antonyak *et al.*, J. Biol. Chem., 273:2817-22 [1998]; and Logan *et al.*, Mol. Cell. Biol., 7:5784-90. [1997]). It has long been appreciated that c-Jun promotes proliferation (Angel and M. Karin, Biochim. Biophys. Acta 1072:129-57 [1991]) and both cells lacking c-Jun and cells expressing its non-phosphorylatable dominant negative counterpart, c-Jun(Ser63A,Ser73A), display well-defined growth defects (Behrens *et al.*, *supra*); and Wisdom *et al.*, *supra*). A recent study has provided evidence suggesting that c-Jun controls cell cycle progression in a p53-dependent manner (Waldman *et al.*, Cancer Res., 55:5187-90 [1995]). The JNK pathway has also been implicated in the transformation of pre-B cells by *Brc-Abl* and in the transformation of fibroblasts by the *Met* oncogene (Raitano *et al.*, Proc. Natl. Acad. Sci. USA 92: 11746-11750 [1995]; Rodrigues *et al.*, EMBO J. 16:2634-2645 [1997]). In addition, both EGF-dependent proliferation and anchorage-independent growth of A549 cells can be prevented by either stable expression of c-Jun(Ser63A,Ser73A) or by addition of JNK antisense oligonucleotides (Bost *et al.*, J. Biol. Chem. 272:33422-29 [1997]; and Bost *et al.*, [1999a], *supra*, Mol. Cell. Biol., 19:1938-1949). While most of the effects of JNK described above have been attributed to its phosphorylated state, it has recently become apparent that non-phosphorylated JNK (that lacking kinase activity) also contributes to the regulation of its substrates. In contrast to activated JNK, which serves to stabilize

and enhance the transcriptional activity of its substrates, non-phosphorylated JNK appears to lead to ubiquitination and degradation of its substrates (Fuchs *et al.*,Oncogene 13:1531-35 [1996]; Fuchs *et al.*, Genes Dev., 12:2658-63 [1998a]; and Fuchs *et al.*, Proc. Natl. Acad. Sci. USA 95:10541-6 [1998b]). Thus, both inactive and active JNK are likely to play a role in regulating a variety of cell processes including growth, apoptosis and transformation.

The present invention provides compositions and methods contemplated for use in the treatment of certain cancers and for the screening of compounds that may mimic or augment JNK2AS activity. Although the present invention is not limited to any particular mechanism, to investigate the role of JNK in regulating tumor cell growth, highly specific JNK antisense oligonucleotides (JNKAS) were utilized, to reduced the expression of *JNK1* and *JNK2* (Bost *et al.*, [1997], *supra*; Bost *et al.*, [1999a], *supra*; and Xu *et al.*, J. Biol. Chem., 273:33230-8 [1998]). A preliminary survey of several different cell lines revealed that JNKAS treatment had little or no effect on the growth of most of the cell lines examined, but three cell lines displayed marked growth inhibition in response to such treatments (Table 1, below).

TABLE 1. Inhibition of Growth by JNK Antisense Oligonucleotides in Different Human Tumor Cell Lines.

Tumor Cell Line	Growth Inhibition by JNKAS Treatment[a]	p53 Status
PC3 prostate carcinoma	+++	null
A549 lung carcinoma	+/-	wild-type
T98G glioblastoma	++	mutant
HeLa cervical carcinoma	+++	null
RKO colorectal carcinoma	-	wild-type
MCF-7 breast carcinoma	-/+	wild-type
HCT116 colorectal carcinoma	+/-	wild-type

[a] Cells were treated with a combination of JNK1AS and JNK2AS (0.2 mM each), and viable cell mass was measured 3-5 days after the treatment using MTS dye reduction assay as described in the Examples section.

Interestingly, the growth inhibitory response to JNKAS treatment appeared to correlate with the cell's p53 status, in that suppression of growth occurred only in cells harboring mutations in p53 (Bost *et al.*, Prostate 38:321 [1999b]). The development of the study invention involved further investigations into the possibility that p53 might directly influence the response of tumor cells to JNKAS treatment. To this end, derivatives of MCF-7 and RKO cells in which p53 function was abrogated through expression of viral E6 oncoprotein were used (Rodrigues *et al.*, EMBO J., 16:2634-2645 [1997]), as well as HCT116 $p53^{-/-}$ cells, where *p53* gene was disrupted through homologous recombination (Bunz *et al.*, Science 282:1497-501 [1998]). The demonstration that the loss of p53 function in these cells results in growth inhibition and apoptosis following JNKAS treatment provides additional evidence indicating that inability to elevate $p21^{Cip1/Waf1}$ levels

contributes to the enhanced sensitivity of p53-deficient cells to JNKAS. These findings support an important role for the JNK pathway in maintenance of normal tumor cell growth in the absence of p53 function and suggest the utility of strategies aimed at eliminating JNK for treatment of tumors harboring mutant p53.

Over the past several years, the functional role of the JNK pathway has been extensively investigated. The vast majority of studies have relied on over expression of mutant forms (dominant-negative or constitutively active) of various upstream signaling components to modulate JNK kinase activity. Although informative, this approach can produce confounding results for several reasons. First, most upstream JNK regulators are also implicated in other signaling pathways (*e.g.* p38, ERK and others) and thus alterations in these pathways can contribute to the effects observed. Second, considerable redundancy exists in the upstream components (*e.g.* both MEKK4 and MEKK7 phosphorylate and activate JNK), and therefore interference of one component's activity often leads to only partial inhibition of JNK activation. Finally, such approaches described above do not alter JNK protein levels, and therefore do not allow the investigation of the importance of changes in basal JNK activity or other potential functions of the protein unrelated to kinase activity. This is particularly important given that, in the absence of stress, non-phosphorylated JNK has recently been implicated in down-regulating the expression of its substrates, targeting them to degradation (Fuchs *et al.*, [1996], *supra*; Fuchs *et al.*, [1998a], *supra*; and Fuchs *et al.*, [1998b], *supra*; Fuchs *et al.*, Oncogene 12:2558-63. [1998c]). While the recent generation of JNK1, *JNK2*, and *JNK3* knockout mice provides the opportunity to address the roles of specific JNK isoforms in normal mouse cells and tissues, such model systems are not currently available for human cells. Nor do they address the importance of JNK in regulating tumor cell growth. The antisense strategy utilized here avoids many of the drawbacks noted above, and offers several distinct advantages. It produces significant and isoform-specific inhibition of basal *JNK* mRNA and protein expression in human cells without perturbing other components of the pathway. The availability of AS oligonucleotides highly specific for *JNK1* and *JNK2* allows investigation into differential roles of these genes. Using this strategy the growth regulatory functions of JNK1 and JNK2 in otherwise unstressed cells were investigated during the development of the present invention. These

experiments have provided evidence suggesting that JNK2 is required for growth and homeostasis of tumor cells lacking p53 function, as elimination of *JNK2* expression in p53-deficient cells results in their apoptosis (Figures 4-6). This role appears not to be shared by JNK1. However, early experiments have shown that the JNK1 protein has a longer half-life than JNK2 protein, and therefore it remains possible that the more efficient elimination of JNK2 accounts for its greater inhibitory effects.

The ability of JNK to bind to and phosphorylate p53 was reported several years ago (Hu *et al., supra*; Milne *et al., supra*). More recently, several reports have appeared indicating that JNK serves opposing roles in regulating the stability of the p53 protein under normal growth conditions and during stress (Fuchs *et al.*, [1998a], *supra*; Fuchs *et al.*, [1998b], *supra*). Finally, it was recently shown that p53 transcription is subject to repression by activated c-Jun, a major known substrate of JNK (Schreiber *et al.*, Genes Dev., 13:607-19 [1999]). Taken together, these observations demonstrate important links between the activities of JNK and p53. Experiments conducted during the development of the present invention demonstrated that JNK2AS treatment leads to growth inhibition and apoptosis of RKO, MCF-7 and HCT116 human tumor cells in a p53-dependent manner, providing evidence for yet another link between these stress-regulated pathways. The greater sensitivity of p53-deficient tumor cells to JNK2AS treatment is not restricted to these cell types as it was observed that other human tumor cell lines with null or mutant p53 status display greater growth inhibition/cytotoxicity following JNKAS treatment relative to cells with normal p53 function (Table 1; Bost *et al.*, [1999b], *supra*). Whether re-introduction of wild type p53 into tumor cells with mutant/null p53 status can alter their response to JNK antisense treatment remains to be investigated.

p53 can act in two opposing directions to influence a cell's sensitivity to stressful conditions: it can promote apoptosis in response to certain cytotoxic agents (*e.g.* hydrogen peroxide) while conferring protection against the cytotoxic effects of others (*e.g.* UVC irradiation and TNF-α; *See e.g.*, Brown and Wouters, Cancer Res., 59:1391-1399 [1999]; Bunz *et al.*, *supra*). The factors contributing to these seemingly disparate functions of p53 are far from clear, but recent studies have provided strong evidence that the protective influence of p53 is mediated

largely through its ability to up-regulate $p21^{Cip1/Waf1}$ expression (Gorospe *et al.*, Mol. Cell. Biol. 18:1400-7 [1998]; Gorospe *et al.*, Gene Expression 7:337-385 [1999]). Consistent with this view it was observed that MCF-7, RKO, and HCT116 cells with normal p53 function responded to the JNK2AS treatment with induction of $p21^{Cip1/Waf1}$, while the p53-deficient derivatives did not (Figure 8). Although JNK1AS treatment resulted in induction of $p21^{Cip1/Waf1}$ expression (Figure 8A), this effect was less pronounced compared with that seen in JNK2AS-treated cells, and $p21^{Cip1/Waf1}$ protein levels were induced to a much lesser extent (data not shown). These results, taken together with the absence of marked growth suppression following JNK1AS treatment (Figures 3 and 7), suggest that JNK2 may have specific function(s) which are not shared by JNK1. That $p21^{Cip1/Waf1}$ expression might be important in contributing to survival of JNK2AS-treated cells was supported by additional studies in HCT116 cells in which targeted disruption of the $p21^{Cip1/Waf1}$ gene was likewise associated with enhanced apoptosis following JNK2AS treatment (Figure 9). Based on these observations, the following model is proposed (however, it is not necessary to understand the underlying mechanisms in order to use the present invention).

Tumor cells require JNK2 for normal growth and the depletion of JNK2 in cells with normal p53 function by the JNK2AS treatment perturbs normal homeostasis triggering a "stress response". This results in the induction of $p21^{Cip1/Waf1}$ which promotes survival. The lack of p53 in the E6-expressing and $p53^{-/-}$ cells prevents induction of $p21^{Cip1/Waf1}$, depriving them of this important protective factor, and therefore leads to growth suppression and apoptosis. It is not clear, however, how the death program is initiated or who the important mediators of this response are. It is also important to note that not all p53-deficient cells undergo apoptosis in response to JNK2AS treatment. T98G glioblastoma cells, for example, do not die, but rather undergo arrest in the S and G2 phases of the cell cycle (see, *e.g.*, example 7). However, unlike p53-deficient cells used in this study, T98G cells show marked induction of $p21^{Cip1/Waf1}$ expression following JNK2AS treatment (see, *e.g.*, example 8), more so in fact than parental MCF-7, RKO, and HCT116 cells. It is likely that the high levels of $p21^{Cip1/Waf1}$ contribute to the cell cycle arrest, as the importance of $p21^{Cip1/Waf1}$ in mediating G2 arrest has recently been established (Bunz *et al.*, *supra*).

In summary, the examples of the present invention show that the JNK pathway plays an important role in regulating growth of tumor cells and may in fact contribute to cellular transformation (Bost *et al.*, [1997], *supra*; Bost *et al.*, [1999a], *supra*; Bost *et al.*, [1999b] *supra*). Furthermore, the examples of the present invention demonstrate that JNK (JNK2 in particular) is important for growth of human tumor cells lacking functional p53.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for the use of mRNA sequences having homology to JNK2 sense strand mRNA. In one embodiment, the JNK2AS strands are used for the reduction or elimination of p53-deficient cancers. In an other embodiment the JNK2AS oligonucleotide strands are used in conjunction with agents suspected of reducing or inhibiting p53 activity thereby making the cancer p53-deficient. Another aspect of the present invention is to provide methods for the introduction of JNK2AS oligonucleotide into cells *in situ*. Additionally, the present invention relates to methods for the screening of compounds that are suspected of mimicking or augmenting JNK2AS activity. In this regard, the description of the invention is divided into (a) a review of the principles of antisense technology, (b) *in situ* transfection methods, (c) methods for selective compound screening and (d) methods for rendering cells p53-deficient.

A. Antisense Technology

Antisense technology is a novel drug discovery method. Antisense drugs work at the genetic level to interrupt the process by which disease-causing proteins are produced. Proteins play a central role in virtually every aspect of human metabolism. Almost all human diseases are the result of inappropriate protein production (or disordered protein performance). This is true of both host diseases (*e.g.*, cancer) and infectious diseases (*e.g.*, AIDS). Traditional drugs are designed to interact with protein molecules throughout the body that support or cause diseases. Antisense drugs (*i.e.*, antisense mRNA strands in a pharmacologically suitable carrier) are designed to inhibit the production of disease-causing proteins. Additionally, in the present invention, antisense drugs are designed to inhibit cellular pathways leading to the reduction of proliferation and/or the induction of

apoptosis. In this regard, antisense drugs can be designed to treat a wide range of diseases including infectious, inflammatory and cardiovascular diseases and cancer and have the potential to be more selective and, as a result, more effective and less toxic than traditional drugs.

How do antisense drugs work? Although the present invention is not limited to any particular mechanism, the information necessary to produce proteins in cells is contained in genes. Specific genes contain information to produce specific proteins. The information required for the human body to produce all proteins is contained in the human genome and its collection of more than 100,000 genes. Genes are made up of DNA which contains information about when and how much of which protein to produce, depending upon what function is to be performed.

The DNA molecule is a "double helix" -- a duplex of entwined strands. In each duplex, the bases or nucleotides (Adenine, Thymidine, Guanine, Cytosine) are weakly bound or "paired" by hydrogen bonds to complementary nucleotides on the other strand (A to T, G to C). Such highly specific complementary base pairing is the essence of information transfer from DNA to its intermediary, messenger RNA (mRNA), and carries the information, spelled out by the specific sequences of bases, necessary for the cell to produce a specific protein.

During transcription of information from DNA into mRNA, the two complementary strands of the DNA partly uncoil. The "sense" strand separates from the "antisense" strand. The "antisense" strand of DNA is used as a template for transcribing enzymes which assemble mRNA -- a process called "transcription". Then, mRNA migrates into the cell where other cellular structures called ribosomes read the encoded information, its mRNA's base sequence, and in so doing, string together amino acids to form a specific protein. This process is called "translation."

Antisense drugs are complementary strands of small segments of mRNA. To create antisense drugs, nucleotides are linked together in short chains (called "oligonucleotides"). Each antisense drug is designed to bind to a specific sequence of nucleotides in its mRNA target to inhibit production of the protein encoded by the target mRNA. By acting at this earlier stage in the disease-causing process to prevent the production of a disease-causing protein or block the production of a

protein or proteins that permit the survival of diseased cells (*e.g.*, cancer cells), antisense drugs have the potential to provide greater therapeutic benefit than traditional. Additionally, antisense drugs have the potential to be much more selective or specific than traditional drugs, and therefore more effective, because they bind to mRNA targets at multiple points of interaction at a single receptor site. Traditional drugs usually bind at only two points of interaction.

Antisense mRNA strands and drugs may be produced by any number of methods known to those in the art. For example, mRNA antisense strands may be made utilizing PCR techniques (*See e.g.*, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, all of which are herein incorporated herein by reference). Additionally, antisense mRNA strands and drug may be made utilizing oligonucleotide synthesizers. Furthermore, anti-sense oligonucleotides are commercially available (ISIS).

B. *In situ* Transfection for the Treatment of Tumors

In situ transfection is the process of inserting an oligonucleotide into a cell or cells in a patient or host organism. Several methods are available to perform *in situ* transfection. Viruses may be engineered to express the desired antisense oligonucleotide (Lattime *et al., supra*). Viral transfection has the advantage of being easily administered. Additionally, expression promoters may be cell or tissue type specific thereby allowing for directed administration of the oligonucleotide encoding JNK2AS oligonucleotide. In this regard, retroviral infection of cells or tumors with genetically engineered retroviruses may also be used to introduce transgenes into an animal cell. For example, blastomeres have been used as targets for retroviral infection (Jaenisch, Proc. Natl. Acad. Sci USA 73:1260-1264 [1976]). Transfection is typically achieved using a replication-defective retrovirus carrying the transgene (Jahner *et al.*, Proc. Natl. Acad. Sci. USA 82:6927-6931 [1985]; Van der Putten *et al.*, Proc. Natl. Acad Sci USA 82:6148-6152 [1985]). The advantages of retroviral infection methods include the ease of transfection and the insertion of a single copy of the transgene, which is flanked by the retroviral long terminal repeats (LTRs), into the chromosome.

Another method of *in situ* transfection include the direct injection (microinjection) into the tumor of the oligonucleotide encoding JNK2AS oligonucleotide or the direct injection of JNK2AS oligonucleotide (see Example 5)

(Palmiter, Ann. Rev. Genet., 20:465-499 [1986]). Technical aspects of the microinjection procedure and important parameters for optimizing integration of nucleic acid sequences have been previously described (Brinster *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442 [1985]; Gordon *et al.*, Meth. Enzymol., 101:411-433 [1983]). Cells take up the oligonucleotide (either the vector encoding the JNK2AS oligonucleotide or the mRNA itself). If an expression vector is transfected, the DNA is expressed producing the JNK2AS oligonucleotide. If the JNK2AS oligonucleotide is transfected, no expression is necessary. Said injections may be made one or more times depending on factors such as size of the tumor, location of the tumor, effectiveness of the treatment for the specific tumor, etc.

Yet, other methods for the *in situ* transfection of JNK2AS oligonucleotide or vectors encoding JNK2AS oligonucleotide include particle bombardment and microparticle injection techniques (Johnston and Tang, Genet. Eng. (NY), 15:225-236, [1993]; Yang, Crit. Rev. Biotechnol., 12:335-356 [1992]). In these techniques, oligonucleotides encoding JNK2AS oligonucleotide, or the JNK2AS oligonucleotide itself, is attached to microparticles such as synthetic beads. Such beads would be capable of binding oligonucleotides and be of such size as to be readily taken up by cells.

Patients being treated via the methods listed above would be monitored for disease progression or reduction. One of skill in the art would recognize that detection of the effect of the compound being tested on cancer may be determined according to standard techniques well-known in the art. These techniques include visual inspection, immunohistochemical techniques, reverse PCR and the like. For example, the change in the size of tumors may be monitored using calipers.

Selective inhibition of translation of the gene of interest in tissues and cells of patients or non- animals may be determined using several methods known in the art as well as using methods described herein. For example, expression of the protein product of the gene of interest (*e.g.* JNK2) may be determined using immunohistochemical techniques. Briefly, paraffin-embedded tissue sections are dewaxed, rehydrated, treated with a first antibody which is specific for the polypeptide product of the gene of interest. Binding is visualized, for example, by using a secondary biotinylated antibody which is specific for the constant region of the primary antibody, together with immunoperoxidase and 3,3'-amiobenzidine as a

substrate. Sections may then be stained with hematoxylin to visualize the cellular histology. Antibody binding of tissues and cells which is detected by antibody binding demonstrates expression of the protein product of the gene of interest in these tissues and cells.

Yet another alternative method for the detection of expression of the protein product of the gene of interest is by Western blot analysis wherein protein extracts from different tissues are blotted onto nitrocellulose filters, and the filters incubated with antibody against the protein product of the gene of interest, followed by detection of antibody binding using any of a number of available labels and detection techniques (*See e.g.*, Example 1).

C. Drug Screens

The present invention also contemplates drug screens for compounds that mimic or augment JNK2AS therapy. These drug screens would utilize JNK2AS oligonucleotide control cells. The cells would be p53-deficient transformed cell lines. Additionally, non-human animals may be treated to develop one or more p53-deficient tumors (*See*, Example 5). said transformed cell lines or said treated non-human animals would then i) be left untreated, ii) be treated with JNK2AS oligonucleotide iii) be treated with a compound or compounds suspected of mimicking or augmenting JNK2AS oligonucleotide activity, or iv) be treated with both JNK2AS oligonucleotide and a compound or compounds suspected of mimicking or augmenting JNK2AS oligonucleotide activity. The cells or animals would then be monitored for changes in tumor size, shape and composition. One of skill in the art recognizes that detection of the effect of the compound being tested on cancer may be determined according to standard techniques well-known in the art. These techniques include visual inspection, immunohistochemical techniques, reverse PCR and the like. For example, the change in the size of tumors may be monitored using calipers.

Following initial screening, a compound that appears promising is further evaluated by administering various concentrations of the compound to the treated non-human animals provided herein in order to determine an approximate therapeutic dosing range.

D. Methods for Rendering Cells and Tumors p53-Deficient

The novel and non-obvious aspect of the present invention relates, in part, to the ability of JNK2AS oligonucleotide to reduce or eliminate p53-deficient transformed cells and tumors through apoptotic mechanisms alone or in conjunction with reduced proliferation. In that regard, it is another aspect of the present invention to treat non-p53-deficient cells or tumors with JNK2AS oligonucleotide by making the cells or tumors p53-deficient before and/or simultaneously with JNK2AS treatment. Therefore, the present invention contemplates the use of p53 antisense (p53AS) mRNA to reduce or eliminate p53 function in cells or tumors. P53AS mRNA may be delivered to said cells or tumors via the mechanisms detailed above in part B (*i.e., in situ* transfection) of this section.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be read as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); IM (intramuscular); IP (intraperitoneal); IV (intravenous or intravascular); SC (subcutaneous); H_2O (water); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); nM (nanomolar); U (units); V (volts); MW (molecular weight); µCi (microcurrie); sec (seconds); min(s) (minute/minutes); hr(s) and h (hour/hours); ab (antibody); FITC (fluoroscein isothiocyanate); HCl (hydrochloric acid); OD_{280} (optical density at 280 nm); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PCR (polymerase chain reaction); PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); RT-PCR (reverse transcription PCR); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); EDTA (Ethylenediaminetetraacetic Acid); w/v (weight to volume); v/v (volume to volume); Millipore (Millipore, Bedford, MA); ISIS (ISIS Pharmaceuticals, Carlsbad, CA); Bio-Rad (BioRad, Richmond, CA); and Sigma (Sigma Chemical

Co., St. Louis, MO); Biofluids (Biofluids, Rockville, MD); Hyclone (Hyclone Laboratories, Logan, UT); Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA); Pharmacia (Pharmacia Biotech, Uppsala, Sweden); Molecular Dynamics (Molecular Dynamics, Sunnyvale, CA); NEN (NEN Life Science Products, Boston, MA); Promega (Promega, Madison, WI); Boehringer Mannheim (Boehringer Mannheim, Indianapolis, IN); Becton Dickinson (Becton Dickinson, San Jose, CA); Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA); Clontech (Clontech, Palo Alto, CA); Tel-Test (Tel-Test, Friendswood, TX); PharMingen (San Diego, CA); and Life Technologies (Life Technologies, Bethesda, MD).

EXAMPLE 1

Specific Inhibition of JNK Expression by JNKAS

To verify that the lipofection procedure used here resulted in high-efficiency oligonucleotide uptake in MCF-7 and RKO cells, the control fluorescein (FITC)-labeled phosphorothioate oligonucleotide ISIS13193 was employed. Cell were cultured as follows: Human breast carcinoma MCF-7 neo and MCF-7 E6 cells, a gift from Dr. A. J. Fornace, were cultured in RPMI 1640 (Life Technologies). Human colorectal carcinoma RKO neo and RKO E6 cells, a gift from Dr. M. Kastan, were cultured in MEM (Life Technologies). HCT116 and their $p21^{-/-}$ and $p53^{-/-}$ derivatives (8, 42), a gift from Dr. B. Vogelstein, were grown in McCoy's 5A medium (Biofluids). The medium was supplemented with 10% FCS (Hyclone), non-essential amino acids, and antibiotics. For these examples, cells were treated with 0.3 μM oligonucleotides in the presence of 10 μg/ml of Lipofectin reagent (Life Technologies) as described known in the art. To determine the efficiency of the oligonucleotide transfection into MCF-7 and RKO cells, a fluorescein (FITC)-labeled phosphorothioate control oligonucleotide, ISIS13193 5'-TCC CGC CTG TGA CAT GCA TT-3' (SEQ ID NO:5), was used. At the indicated times following lipofection with the FITC-oligonucleotide, cells were fixed in 3.7% formaldehyde, and images were taken using a Carl Zeiss LSM 410 confocal microscope (488-nm laser wavelength; 40X/1.3 magnification; pinhole size 20 nm). Shown in Figure 1 is green fluorescence, visualized 24 h after lipofection with 0.4 mM ISIS13193. Virtually 100% of cells showed uptake of the transfected

oligonucleotide. Similar results were obtained at the 12- and 48-h time points and over an extended dose-response range (0.1- 0.5 μM oligonucleotide). Mock-lipofected cells did not excite any fluorescent signal (data not shown). No differences in the efficiency of oligonucleotide uptake were seen between p53-deficient (E6-expressing) and p53-proficient (expressing a neo-containing vector control) cells.

Next, the effect of JNK1 and JNK2 antisense oligonucleotides (JNK1AS and JNK2AS, respectively) on *JNK* expression in MCF-7 and RKO cells was investigated. The oligonucleotides used in this study were synthesized at ISIS. The sequence of the oligonucleotides used are as follows: JNK1AS (ISIS12539) 5'-CTC TCT GTA GGC CCG CTT GG -3' (SEQ ID NO:1); JNK2AS (ISIS 12560) 5'-GTC CGG GCC AGG CCA AAG TC-3' (SEQ ID NO:2); JNK1Scr (ISIS14321) 5'-CTT TCC GTT GGA CCC CTG GG-3' (SEQ ID NO:3); and JNK2Scr (ISIS14319) 5'-GTG CGC GCG AGC CCG AAA TC-3' (SEQ ID NO:4). All oligonucleotides were 2'-O-methoxyethyl (MOE) chimeric antisense oligonucleotides containing five MOE/phosphodiester residues flanking a 2'-deoxynucleotide/phosphorothioate region. Cells were treated either with 0.3 μM of JNK1AS or JNK2AS individually, or with JNK1AS and JNK2AS in combination (0.15 μM each). Scrambled sequence oligonucleotides (JNK1Scr and JNK2Scr) were used at the same concentrations and served as controls. Both antisense treatments led to >95% reduction in the corresponding mRNA levels (Figure 2A), while neither mock lipofection nor treatment with scrambled oligonucleotides had any effect on *JNK1* or *JNK2* mRNA expression.

Western analysis of JNK protein expression revealed a corresponding reduction in the levels of JNK protein in JNKAS-treated cells (Figure 2B). For Western analysis, total protein was extracted using whole-cell extract buffer and protein concentration was quantified by the Bradford assay. Protein samples (25-50 μg) were separated in 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). JNK proteins were identified using C-571 anti-JNK primary antibodies (Santa Cruz Biotechnology) and reactive bands were visualized using the enhanced chemiluminescence detection system (NEN). Since differential splicing of both *JNK1* and *JNK2* mRNAs results in the production of 46-kDa and 54-kDa isoforms, and there is a significant cross-

reactivity between the available JNK1 and JNK2 antibodies, one cannot with certainty, distinguish between the JNK1 and JNK2 isoforms. However, based on these observations and those of others, it appears that the slower-migrating proteins ($p54^{JNK}$) are comprised primarily of JNK2 isoforms, whereas the faster-migrating forms ($p46^{JNK}$) contain mostly JNK1. Hence, JNK1AS was more effective in eliminating the $p46^{JNK}$ isoform, while JNK2AS was more effective in eliminating the $p54^{JNK}$ form (Figure 2B).

An immunocomplex kinase assay was used to examine basal and UVC-induced JNK activity in mock-, JNKAS- and JNKScr-treated cells (Figure 2C). To perform the assay, cells were irradiated with 40 J/m^2 UVC 24 h following the lipofection, and 30 min later washed with cold PBS and suspended in whole-cell extract buffer. The JNK kinase assay was performed with a fusion protein containing glutathione linked to the 1-222 fragment of human c-Jun (GST-cJun) as substrate, as known in the art. Briefly, 50 μg of cell lysates were incubated for 3 hours at 4°C with 10 μg of GST-cJun bound to Glutathione Sepharose-4B (Pharmacia). After washing three times in WCE buffer and once in kinase reaction buffer (20 mM HEPES pH 7.7, 20 mM $MgCl_2$, 20 mM α-glycerophosphate, 20 mM p-nitrophenyl phosphate, 0.1 mM sodium vanadate, 2 mM DDT) the beads were incubated with 30μl of kinase reaction buffer containing 20 μM ATP and 5 μCi [g-^{32}P]-ATP for 30 min at 30°C. The reaction was stopped by addition of Laemmli sample buffer, samples were boiled for 5 min and resolved by electrophoresis through 12% polyacrylamide-SDS gels (SDS-PAGE). The [γ-^{32}P]-phosphorylated GST-cJun was quantified by using a PhosphorImager™ (Molecular Dynamics). No significant JNK activity was detectable in any of the treatment groups of either wild type or p53-deficient MCF-7 and RKO cells in the absence of stress. Consistent with the reduction in JNK protein levels, UVC-induced JNK activation was markedly reduced in both wild type and p53-deficient MCF-7 and RKO cells treated with a combination of JNK1AS and JNK2AS (labeled JNKAS, Figure 2C). Neither mock lipofection nor treatment with scrambled oligonucleotides affected the JNK kinase activity.

Taken together, the experiments described above demonstrate that, using JNKAS, a significant reduction in *JNK* expression, and therefore directly investigate the roles of JNK1 and JNK2 in regulating the growth of p53-proficient and p53-deficient MCF-7 and RKO cells can be achieved.

EXAMPLE 2

Growth Inhibition by JNKAS is Dependent on the p53 Status

Growth of untreated and JNKAS-treated cells was monitored using an MTS dye reduction assay. Cells were seeded in 96-well cluster plates at a density of 15 x 10^3 cells/well and transfected with AS oligonucleotides as described above. At the designated times, viable cell mass was measured by detection of 3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) dye reduction at 490 nm as described in the manufacturer's protocol (Promega). All viability assays were carried out in triplicate. JNKAS treatment had little to no effect on the growth of wild type (neo) MCF-7 and RKO cells (Figure 3). A small transient reduction in the growth of JNK2AS-treated MCF-7 cells was apparent at 24-48 h following lipofection, but viable cell mass did not differ from that of control cultures by 72 h post treatment. No significant effect on growth was observed in wild type RKO cells with any treatment. In contrast, p53-deficient derivatives (E6) of both MCF-7 and RKO cells displayed a marked reduction in cell viability following treatment with JNKAS; the growth inhibitory effect was greater for JNK2AS than for JNK1AS. Growth suppression by JNK2AS was most pronounced in p53-deficient RKO cells, where viability was reduced >70% by 48 h following treatment with the oligonucleotide.

Inhibition of JNK2 expression results in apoptosis of p53-deficient cells. To determine the cause for the decline in growth of JNKAS-treated cell populations, experiments were conducted to first examine whether DNA synthesis was altered after application of JNKAS. No differences in BrdU incorporation relative to the number of viable cells among any of the treatment groups were observed (data not shown), thus the reduction in growth of p53-deficient cells could not be explained by an inhibition of DNA replication. Next the possibility that the decrease in cell number reflected selective death in JNKAS-treated cultures was tested. Shown in Figure 4 is the morphology of parental and p53-deficient MCF-7 and RKO cells

with or without JNKAS treatment. The p53-deficient (E6) lines treated with JNK2AS revealed features consistent with apoptosis including cell rounding, membrane blebbing, and detachment from the tissue culture dish (Figure 4). These effects were also evident to some degree in JNK1AS-treated cultures, but were completely absent in p53-deficient cells subjected to either mock treatment or treatment with scrambled oligonucleotides. They were also absent in p53-proficient (neo) cells regardless of treatment.

Biochemical evidence of apoptosis in the p53-deficient cells was obtained using an ELISA assay for detection of cytoplasmic histone-associated DNA fragments (Figure 5). Apoptosis was assessed using an ELISA Cell Death Detection assay (Boehringer Mannheim) following the protocol provided by the manufacturer. Oligonucleosomal fragments, released from the nuclei after internucleosomal degradation of genomic DNA were detected in cytoplasmic fractions of 2,000 cells. For cell cycle analysis, cells were collected 24 h following treatment, fixed in 70% ethanol, and stained with 1 μg/ml propidium iodide (Cellular DNA Flow Cytometric Analysis kit, Boehringer Mannheim). DNA content was analyzed on FACScalibur™ flow cytometer (Becton Dickinson). The percentages of cells in various phases of the cell cycle were determined using Becton Dickinson ModFitLT software. Both E6-expressing MCF-7 and RKO lines exhibited significant induction of apoptosis 24 h following JNK2AS treatment. With this assay, some evidence of DNA degradation in JNK2AS-treated RKO neo cells as well as in RKO E6 cells treated with JNK1AS was also observed, but quantitatively it was not nearly as great as that seen in JNK2AS-treated RKO E6 cells.

Consistent with the biochemical analysis, FACS analysis of RKO E6 cells as early as 24 h after treatment revealed the presence of a sub-G1 cell population in cultures treated with JNK2AS alone, or with JNK1AS and JNK2AS in combination (Figure 6). No sub-G1 peak was evident in RKO E6 cells subjected to any of the control treatments, or in the RKO neo cells regardless of treatment. The number of apoptotic cells in JNK2AS-treated RKO E6 cultures further increased with time, and by 48 h post-treatment had reached 14% (data not shown). Taken together, these experiments indicate that JNK2AS treatment results in apoptosis of p53-deficient MCF-7 and RKO cells, thus accounting largely for the growth suppressive effects described above (Figure 3).

EXAMPLE 3

Effects of JNKAS Treatment on HCT116 $p53^{-/-}$ Cells

Although expression of the E6 oncoprotein is a well recognized way to inhibit p53 function, it remained possible that the findings in RKO E6 and MCF-7 E6 cells could reflect other function(s) of E6 protein. To address this possibility another model of p53 deficiency, HCT116 human colorectal carcinoma cells rendered p53-null by a somatic knock-out procedure was employed. Consistent with the observations with E6-expressing RKO and MCF-7 cells, HCT116 $p53^{-/-}$ cells displayed significantly greater sensitivity (Student's test $p < 0.003$) to JNK2AS treatment than parental HCT116 cells with normal p53 status (Figure 7).

EXAMPLE 4

Induction of $p21^{Cip1/Waf1}$ in p53-Proficient and p53-Deficient Cells Following JNKAS Treatment

The finding that inhibition of *JNK* expression, especially *JNK2*, resulted in preferential apoptosis of p53-deficient cells suggests that cells with intact p53 signaling possess a protective mechanism that is unavailable to their p53-deficient counterparts. Early experiments showed that the ability of cells to elevate expression of the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1/Waf1}$ following exposure to cellular stress leads to enhanced survival. Given that p53 is an important regulator of $p21^{Cip1/Waf1}$ expression during stress, and that perturbations in *JNK* expression could constitute a stress to growing cells, $p21^{Cip1/Waf1}$ mRNA levels in JNKAS-treated cells were examined. Expression of $p21^{Cip1/Waf1}$ was detected by hybridization with an 46-base oligonucleotide specific for human $p21^{Cip1/Waf1}$ (Integrated DNA Technologies). Variations in loading and transfer among samples were monitored by hybridizing to a 29-base oligonucleotide complementary to 18S RNA (Clontech). Hybridization and washes were performed by the Church and Gilbert method known in the art, and radioactive signals were quantified using a PhosphorImager™ (Molecular Dynamics).

Total RNA was isolated using Stat-60 solution (Tel-Test). RNA samples (20 μg) were denatured, size-separated by electrophoresis in 1.2% agarose-formaldehyde gels, and transferred onto GeneScreen Plus membranes (NEN). For the detection of *JNK1* and *JNK2* mRNAs, the respective cDNAs were excised from

3xHA-JNK1-SRα3 and 1xHA-JNK2-SRα3, and labeled with [α-^{32}P]dATP using a random primer labeling kit (Boehringer Mannheim). Treatment with JNKAS (especially with JNK2AS) resulted in induction of *p21$^{Cip1/Waf1}$* mRNA in parental MCF-7, RKO, and HCT116 cells. In contrast, no elevation in *p21$^{Cip1/Waf1}$* mRNA was evident in p53-deficient MCF-7 E6, RKO E6, and HCT116 p53$^{-/-}$ cells (Figure 8A). That the increase in *p21$^{Cip1/Waf1}$* mRNA results in increased p21$^{Cip1/Waf1}$ protein expression is shown in Figure 8B, where p21$^{Cip1/Waf1}$ protein levels were examined 24 h following JNK2AS treatment. All three p53-proficient cell lines showed an increase in p21$^{Cip1/Waf1}$ protein expression, but this p21$^{Cip1/Waf1}$ induction was not seen in the p53-deficient derivatives.

Finally, to more directly examine the influence of p21$^{Cip1/Waf1}$ on cell survival following JNKAS treatment, HCT116 colorectal carcinoma cells in which the p21$^{Cip1/Waf1}$ genes were disrupted by homologous recombination were tested. Wild type HCT116 cells (+/+), and HCT116 cells in which one (+/-) or both (-/-) *p21$^{Cip1/Waf1}$* alleles were disrupted, were examined for apoptosis by flow cytometry following treatment with antisense oligonucleotides (Figure 9). No significant apoptosis was evident in p21$^{+/+}$ cells regardless of treatment (1.5% in control populations *vs* 3% in JNKAS-treated cultures). p21$^{-/-}$ cells exhibited higher basal apoptosis compared to the p21$^{+/+}$ parental cell lines (4% *vs* 1.5%) perhaps reflecting the importance of p21$^{Cip1/Waf1}$ for normal cell homeostasis. Neither mock lipofection nor treatment with JNKScr or JNK1AS altered this level. However, treatment of the p21$^{-/-}$ cells with JNK2AS resulted in a substantial increase in the sub-G1 population within 24 h after treatment (Figure 9). An intermediate level of apoptosis was observed following JNK2AS treatment of cells containing one functional p21$^{Cip1/Waf1}$ allele (+/-), suggesting that the relative level of p21 expression might be important in determining the outcome. These results further support the hypothesis that p21 contributes to the survival of cells following JNKAS treatment.

EXAMPLE 5

p53-Deficient Tumors are Apoptoticly Reduced by JNK2AS Methods in Mouse Model

Xenographs of a subclone of PC3 cells were selected following transfection with an empty-vector, pLHCX (21), that are virtually 100% tumorigenic and rapidly growing in athymic mice (Figure 10A). Following the appearance of visible measurable tumors (average 180 mm^3), systemic treatment was begun (arrow, Figure 10A) by interperitoneal (IP) administration of the various oligonucleotides 6 days/week. For comparison, the effect of cisplatin chemotherapy was also examined. Cisplatin was administered IP weekly on the seventh day at the maximum tolerable dose ($\approx$ 20 % IC_{50}) as determined in preliminary studies (data not shown). Systemic treatment with cisplatin substantially reduced tumor size leading to an integrated average tumor growth curve 57 % of the areas of that for untreated tumors at the end of the 26 day treatment period indicating 43 % size reduction (Figure 10A). Comparison of the results for all data of the growth curve to that of either control curve by analysis of variance (ANOVA), confirmed significant size reduction, $p < 0.0072$. Treatment with antisense lead to an even greater size reduction of 78 %, $p < 0.001$. In contrast, treatment with control oligonucleotide in vehicle made little difference (Figure 10A). Combined antisense and cisplatin treatment lead to only an incremental ($p = 0.089$) increase in arrest to the maximum observed size reduction of 89 %. The final average tumor volume was about 8-fold less than that of the average control tumor (Figure 10A). Owing to the method of volume estimation, ($V \approx \pi/6(1w^2)$), an 8-fold difference corresponds to an average length and width of treated tumors that are half those of control tumors as illustrated in Figure 10C and 10D. Thus, size reduction promoted by antisense treatment is greater than that of cisplatin alone.

If tumor size reduction effects observed here are treatment-related, it would be expected that upon cessation of treatment the reduced tumors would exhibit renewed growth. Figure 10B shows that, following a lag period of approximately one week, the tumors of two groups exhibited a burst in tumor growth leading to final growth rates (slopes) equal to that of the control groups suggesting that, for these groups, viable cells remained in the arrested tumors retaining the ability to grow upon cessation of treatment.

For the antisense JNK treatment group, however, after day 38 of the experiment, there were no remaining intact tumors for valid estimates on tumor volume (Figure 10B). Starting approximately half-way through the treatment period, increasing numbers of tumors developed small darkened spots invariably at the most distal site of the tumor that rapidly ulcerated (via apoptotic cell death, data not shown) leading to progressive and massive cavitation of the tumor. All these cases were excluded from the volume estimates of Figure 10 from the start of the ulceration. Ulceration first began among the antisense-treated animals (Figure 11A). The regression process was tracked by measuring the growth of the diameters of the ulcer cavities (Figure 11B). As the average ulcer sizes of surviving animals approached maximum values, visible tumor mass retreated to a circumferential rim and typically completely vanished. Thus, the plateau values of maximum ulcer diameter of Figure 11B are simply the sizes of the resulting flat sores. In some cases tumor regression proceeded by undermining the skin around a small ulcer mouth leaving the epithelial edges in approximation leading to decreased average diameters with increasing time (Figure 11B) and in one case the ulcer edges reunited to form a healed site. The time course of regression (Figure 11B) also indicates that the process, once started, typically extended well beyond the treatment period suggesting an inflammatory mechanism of continued erosion. Therefore, it is the frequency of initiation of ulceration, *i.e.* Figure 11A, that is taken as the more relevant variable in relating regression to treatment regime. The frequency of ulceration and regression was 40 % for the cisplatin treated animals and 45 % for the combined antisense JNK-cisplatin treated animals. Remarkably, all 15 extant animals of the antisense JNK treated group developed distal ulcers which rapidly led to complete regression (100 %) of tumors. Chi-square analysis of the increased frequency of regression in the antisense JNK related group compared to the next most commonly regressing group, combined antisense JNK-cisplatin, indicates a significant increase, $p = 0.022$. There were no significant differences between either control group and either of the two treatment regimens involving cisplatin. Thus, tumor regression was significantly more common, indeed complete, for antisense JNK treated animals.

Spontaneous ulceration in tumors of control animals also occurred occasionally (Figure 11A), however, this process is distinct. Spontaneous

ulceration occurred late, day ≥ 26, (Figure 11A) when these tumors were very large, > 1000 mm^3 (Figure 10A), whereas treatment-induced ulceration occurred in tumors commonly ≈ 200 mm^3 (*c.f.* Figures 10A and 11A). Spontaneous ulcers involved fewer animals with a maximum incidence of 3 out of 11. On day 43, all remaining intact tumors were large (*c.f.* Figure 10) leading to debilitation and the experiment was terminated.

The antisense compounds used here inhibit steady state protein accumulation and JNK activity for up to 72 h whereas four different control oligonucleotides and mock lipofectin had no effect (data not shown). Unlike other routes of administration of phosphorothioates, intraperitoneal administration of phosphorothioates in aqueous media leads to elevated blood levels that are stable for at least 6 h (data not shown) and to accumulation in xenografts in several systems (data not shown). In order to confirm that systemic treatment of tumor-bearing mice with antisense JNK lead to suppression of JNK in tumor tissue, the JNK activity of treated tumors was examined. Western analysis revealed that JNK1 and JNK2 protein but not β-actin were specifically reduced to nearly undetectable levels (data not shown) and, similarly, that total JNK activity was reduced by 10-fold (Figure 12A). Tumor extracts from untreated or scrambled control-treated animals exhibited elevated JNK activity compared to normal mouse kidney, a site of accumulation of phosphorothioates, as for normally cultured PC3 cells (Figure 12A). These results show that the antisense treatment targeted against JNK1 and JNK2 specifically reduced JNK activity in the xenografts (Figure 12A). On the other hand, tumors from animals treated with the combined antisense JNK-cisplatin regimen exhibit intermediate JNK activities. These intermediate values may be due to an interference in the antisense mechanism by cisplatin or, since cisplatin is a potent activator of JNK, may represent activation of residual JNK protein.

The sum of results suggest that JNK is essential for growth and survival of PC3 cell xenografts. Although the present invention is not limited to any particular mechanism, it may be predicted that if downstream events in the JNK pathway are specifically inhibited by stable expression of c-Jun(S63A, S73A), then the converse result, inhibition of tumorigenesis, would be expected. In this regard, using a clonal line of PC3 cells that express the mutant c-Jun(S63A, S73A), PC3LHCmJun cells, preliminary studies indicated that these cells were entirely unable to develop

tumors in athymic mice. This study was repeated using a separate clonal line of cJun(S63A, S73A)-expressing PC3 cells in both female and male recipient athymic mice (Figure 13). As before, (Figure 10A), empty-vector and control cells rapidly formed tumors in athymic mice whereas c-Jun(S63A, S73A)-expressing cells were entirely unable to develop tumors. All these cell lines are readily cultures in the laboratory over numerous passages and their viability *in vitro* has been studied under a variety of conditions which do not reveal any defect in the absence of cisplatin. These results argue that the JNK pathway is essential for xenograft formation and that, taken together with the high frequency of regression following treatment with antisense JNK, indicate that is required for the survival of PC3 cells as xenografts.

Example 6

Growth Inhibition of T98G Cell Treated with JNKAS

Unlike the p53 null cells discussed above, T98G cells, which contain a mutant version of p53, undergo growth arrest when treated with JNK1AS and JNK2AS. To determine whether inhibition of JNK has an effect on the growth of T98G cells, we assessed the viability of T98G cells over a 24-120 h time period following JNKAS treatment. T98G cells were seeded in 96-well tissue culture plated at a density of 1000 cells/well and transfected with 0.4 μM of the oligonucleotides indicated in Figure 14 on the following day. The MTS dye reduction (Promega) was determined by measurement of optical density at 490 nm. Cell viability was expressed as a percentage of viable cell mass in mock-treated cultures. Cells treated with JNK1AS and JNK2AS exhibit statistically significant inhibition of growth compared with all controls ($p < 0.0001$, Student's t test) and differ among themselves as early as 24 h post-transfection (see asterisk in Figure 14A, $p < 0.001$).

The viability of mock and JNKScr-treated cultures was very similar to that of untreated cells over the entire time course (Figure 14, A and B). In contrast, treatment with either JNK1AS or JNK2AS led to a marked reduction in viable cell mass. Growth suppression following treatment with JNK2AS was more pronounced than seen with JNK1AS treatment (~40% versus 65% that of controls at 24 h post-lipofection, respectively), especially at later time points (~15% versus 75%,

respectively, at 120 h post-lipofection), although statistically significant differences in growth inhibition by both JNKAS were observed as early as 24 h post-lipofection. As determined by direct counting from 24-72 h post-lipofection, the number of viable cells in attached JNK1AS-treated cultures was significantly diminished relative to that of control cultures (Figure 14C). Cells were seeded in six-well cluster plates at 25 x 10^3 cells/cm^2 and treated with 0.4 μM oligonucleotides. Attached cells (open portion of bars) and cells in medium (closed portion of bars) were counted in triplicate 24, 48 and 72 h after transfection. Standard errors were < 10% in all cases. Consistent with the transient loss in JNK expression, JNK1AS-treated cells resumed growth at later times with cell numbers recovering to 70% of that seen in control cultures by day 5 (Figure 14B). JNK2AS-treated cells exhibited a greater loss in cell mass then their JNK1AS-treated counterparts at all times, and, unlike that seen with JNK1AS, their growth did not recover with time. Five days post-lipofection, the viable cell mass of the JNK2AS-treated population was 15% of that seen for control groups (Figure 14B). In summary, although the magnitude of the growth inhibition achieved by JNKAS differed somewhat depending on the method used to assess growth (MTS assay versus cell counting), the general observation of a growth inhibition in response to JNKAS treatment was apparent regardless of the method employed.

To examine the dose dependence for growth inhibition by JNK1AS and JNK2AS treatment, cells were incubated with various amounts of antisense oligonucleotides ranging from 0 to 0.4 μm). The total amount of oligonucleotides added to cells was kept constant by the addition of appropriate concentrations of corresponding JNKScr oligonucleotide. Both JNK1AS and JNK2AS exerted their growth-inhibitory effects in a dose dependent manner (Figure 15). Cell were prepared as above. Cells treated with JNK2AS exhibited a markedly steeper decline in viable cell mass ($^{JNK2}IC_{50}$ = 0.14 μM) compared with cells treated with JNK1AS ($^{JNK1}IC_{50}$ = 0.37 μM). These effects were not apparent in cells treated with similar doses of JNK1S and JNK2S oligonucleotides or in cells receiving 0.4 μM JNKScr. Thus, the inhibitory properties of JNKAS are manifested in a dose-dependent manner and do not reflect cytotoxicity of oligonucleotide treatment *per se* but rather the susceptibility of T98G cells to perturbations in JNK expression.

Example 7

The Growth Inhibition in JNK-depleted Cultures is Associated with Cell Cycle Arrest and Inhibition of DNA Synthesis

Although the present invention in not limited to any particular mechanism, at least two possible cellular responses could account for the reduction in viable cell mass seen following treatment with JNKAS: decreased DNA synthesis and/or selective death of JNKAS-treated cells. First, we examined the treated cells for evidence of apoptosis. JNKAS-treated cultures, particularly JNK2AS-treated cultures, exhibited some morphological alterations frequently seen in cells undergoing apoptosis including fewer mitoses, rounded up cells (Figure 16), and detachment of many cells (up to 30%) from the plate surface (Figure 14C). Detached cells were examined for viability. Up to 90% of detached cells in JNKAS-treated cultures excluded trypan blue and were therefore living cells, although they did not reattach or grow when placed in fresh medium in new dishes (data not shown).

Four additional methods were used to assess apoptosis in JNKAS-treated cells including detection of apoptotic bodies in DAPI-stained cells and various types of flow cytometric analysis: *i.e.* cell cycle distribution of PI-stained cells, detection of BrdUrd incorporation into cellular DNA breaks (APO-BRDU kit, PharMingen), and detection of annexin V-stained populations. Detection of DNA fragmentation by DAPI staining revealed no evidence of apoptotic cells in any treatment groups through 72 h post-lipofection (Figures 17 and 18, data not shown). Interestingly, the structure of nuclei in JNKAS-treated cells differed from that of either mock-treated cultures or cells treated with JNKScr oligonucleotides (Figure 17A). The cause of these alterations is not known, but they may reflect the presence of cells with doubled DNA content that are unable to finish progression through the S phase and mitosis following JNKAS treatment. No cells with condensed or fragmented nuclei indicative of apoptosis were evident in any treatment group. More sensitive methods for assessing apoptosis based on detection of BrdUrd incorporation into DNA breaks (APO-BRDU) (Figure 17B) and detection of specific membrane alterations using annexin V (data not shown) also showed no evidence of apoptosis in any of the treatment groups up to 48 h. Although a small fraction of JNK2AS-treated cells (2.9%) did appear to undergo

apoptosis at 48-72 h (data not shown), this percentage of apoptotic cells is similar to that seen when assaying the negative control provided with the kit (3.3%). Thus, while JNK2 depletion might result in a low level of apoptosis in T98G cells, our results indicate that apoptosis cannot account for the magnitude of the reduction in viable cell mass seen in JNK2AS-treated cultures.

While no evidence for apoptosis of JNKAS-treated cultures was found, JNKAS treatment resulted in profound alterations in the distribution of cells throughout the cell cycle, indicative of cell cycle arrest (Figure 18). In Figure 18A samples were prepared and analyzed as follows. T98G cells were treated with 0.4 μM of the oligonucleotides as labeled in the figure; both attached and detached fractions were collected 24 h after the treatment, fixed stained with propidium iodide and analyzed by flow cytometry. Cultures treated with a combination of JNK1Scr and JNK2Scr (0.2 μM each) are labeled JNKScr. The percentages of cells in G_1, S and G_2/M phases for untreated and control cultures subjected to mock-lipofection or treatment with control oligonucleotides (JNKScr) were similar: $G_1 = 63 \pm 7\%$; $S = 23 \pm 6\%$; $G_2/M = 15 \pm 6\%$ (average data from three independent experiments; Figure 18, A and C). In contrast, lipofection with either JNK1AS or JNK2AS resulted in a 2-fold reduction in the number of cells in the G_1 compartment and an increase in the number of cells in other cell cycle compartments. Interestingly, while JNK1AS-treated cells accumulated in both S and G_2/M phases (35 ± 5.5%; 31 - 4%, respectively), JNK2AS-treated cultures displayed an accumulation of cells exclusively in S phase (50 ± 2%). Additional analysis of distribution of cells through the cell cycle was done using a BrdUrd pulse incorporation assay, which allows detection of S-phase cells by the presence of BrdUrd in their cellular DNA (Figure 18B). For control populations (mock- and JNKScr-treated cells) and for JNK1AS-treated cultures, the percentage of S-phase cells determined with this method (23 and 35%, respectively) were similar to those obtained with PI staining (22 and 34.5%, respectively; data were analyzed using ModFitLT software). However, in the case of JNK2AS-treated cells, BrdUrd incorporation analysis revealed much lower number of S-phase cells than PI staining (28 *versus* 50%). This discrepancy is likely to reflect the fact that T98G cells are not actively progressing though S phase and thus are not incorporating BrdUrd due to JNK2AS inhibitory influence on DNA replication (see below).

To further explore the nature of the growth arrest by JN-KAS, we directly examined DNA synthesis in JNKAS-treated cultures. The experiments were performed as follows: T98G cells were seeded into 96-well tissue culture plates (1000 cells/well) and on the following day treated with various oligonucleotides. Incorporation of [^{3}H]thymidine into polymeric DNA was determined 24 h post-treatment. Results were normalized to relative amounts of viable cells (Figure 14A) and expressed as percentages of values obtained in the untreated population (3 wells/treatment were analyzed in duplicate). The asterisk indicates values for JNK1AS- and JNK2AS treated cells differ significantly from each other (p , 0.001, Student's T test) as well as from control values ($p < 0.001$). As assessed by uptake of [^{3}H]thymidine, DNA synthesis was greatly inhibited in both JNK1AS- and JNK2AS-treated populations (Figure 19). The effect was greater for JNK2AS-treated cultures, which exhibited nearly complete inhibition of DNA synthesis by 24 h post-lipofection (Figure 19A). Interestingly, while DNA synthesis in JNK1AS-treated cells started to recover by 72 h post-lipofection (consistent with the return of JNK1 expression and transient reduction in viable cell mass; Figs. 1 and 4), no such recovery was observed in JNK2AS-treated cultures (Figure 19B). For Figure 19B values in mock-treated cultures were set to 100%. The value labeled "control oligonucleotides" represents the mean of values received from two sense and two scrambled sequence oligonucleotide treatments. Oligonucleotide treatments were performed in triplicate and duplicate samples from each well were assayed for [^{3}H]thymidine incorporation. This is consistent with the lack of recovery in viable cell mass of JNK2AS-treated cultures noted above (Figure 14). Thus, while transient depletion of JNK1 led to a transient inhibition of DNA synthesis and reduction in growth of T98G cells, similar elimination of JNK2 expression led to sustained inhibition of DNA synthesis and permanent growth arrest of T98G cells.

Example 8

Inhibition of Cell Growth by JNKAS is Associated with Inhibition of $p21^{cip1/waf1}$ Expression

Although the present invention is not limited to any particular mechanism, the cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ is believed to play an important role in mediating cell cycle arrest in response to a variety of treatments. Therefore, we investigated its expression in T98G cells following treatment with JNK1AS and JNK2AS. Marked elevation of both $p21^{cip1/waf1}$ mRNA (Figure 20A) and protein (Figure 20B) was detected in JNK2AS-treated cultures. The experiments in this example were performed as follows: For Figure 20A, Northern analysis of $p21^{cip1/waf1}$ gene expression 24 h post-treatment with 0.4 μM of either JNK1AS, JNK2AS, JNK1Scr or JNK2Scr oligonucleotides. Hybridization with a probe recognizing 18 S RNA was used to assess the quality of loading and transfer among samples. Figure 20B is a Western blot analysis for expression of cell cycle regulatory proteins in JNK1AS- and JNK2AS-treated cultures. Figure 20C is a *in vitro* kinase assay for the labeled cell cycle components. Interestingly, this effect was not shared by JNK1AS-treated cells, suggesting that induction of $p21^{cip1/waf1}$ expression may contribute to the specific features of growth arrest observed in JNK2-depleted cultures such as permanent inhibition of DNA synthesis and profound sustained S-phase arrest. No changes in the expression of other cell cycle regulatory proteins were observed following any treatment, except for a slight elevation of $p21^{cip1/waf1}$ protein levels in JNK2AS-treated cultures (Figure 20B). As expected, elevation of $p21^{cip1/waf1}$ levels in JNK2-depleted cells was correlated with a marked inhibition of cyclin-dependent kinase (Cdk2 and Cdc2) activities in JNK2AS-treated cells (Figure 20C).

CLAIMS

1. A vector, comprising a promoter operably linked to a first nucleic acid sequence encoding a second nucleic acid, said second nucleic acid having the sequence selected from SEQ ID NOS: 1 to 5.

2. The vector of Claim 1 in a pharmacologically acceptable carrier.

3. The vector of Claim 1 linked to a peptide.

4. The vector of Claim 3, wherein said linked peptide is a target of a cell surface receptor.

5. Cells comprising the vector of Claim 1.

6. A method, comprising:

1) providing: i) a patient diagnosed with a cancer, and ii) a vector comprising a promoter operably linked to a DNA sequence that encodes nucleic acid having the sequence of SEQ ID NO:2; and

2) administering said vector to said patient.

7. The method of Claim 6 wherein said cancer is characterized as being p53-deficient.

8. The method of Claim 6 wherein said cancer is characterized as having mutant p53.

9. The method of Claim 6, wherein said vector further comprises a cell targeting mechanism.

10. The method of Claim 9, wherein said cell-targeting mechanism is a peptide covalently linked to said vector.

11. A method, comprising:

1) providing: i) a patient diagnosed with a cancer, and ii) an oligonucleotide comprising the sequence of SEQ ID NO:2; and

2) administering said oligonucleotide to said patient.

12. The method of Claim 11 wherein said cancer is characterized as being p53-deficient

13. The method of Claim 11 wherein said cancer is characterized as having mutant p53.

14. A method, comprising:

1) providing: i) one or more compounds, ii) p53-deficient cells, a portion of which are transfected with one or more JNK2AS oligonucleotides so as to define transfected and non-transfected cells;

2) contacting said non-transfected cells with said compound to produce treated, non-transfected cells; and

3) measuring the effect of said compound on said treated non-transfected cells and comparing with effect of JNK2AS treatment on said transfected cells.

15. The method of Claim 14, wherein said p53 deficient cells are cancer cells.

16. The method of Claim 14, wherein said one or more JNK2AS oligonucleotides is SEQ ID NO:2.

FIGURE 1

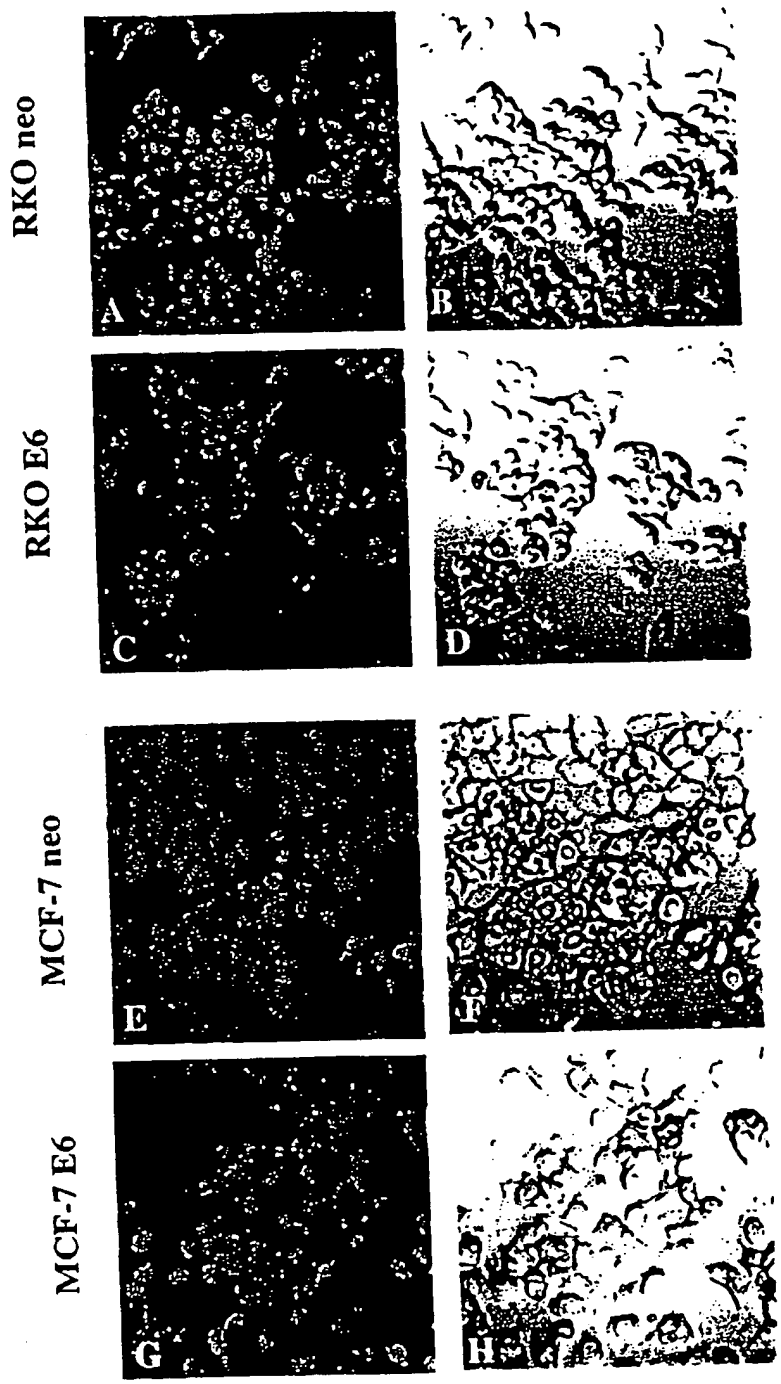


FIGURE 2

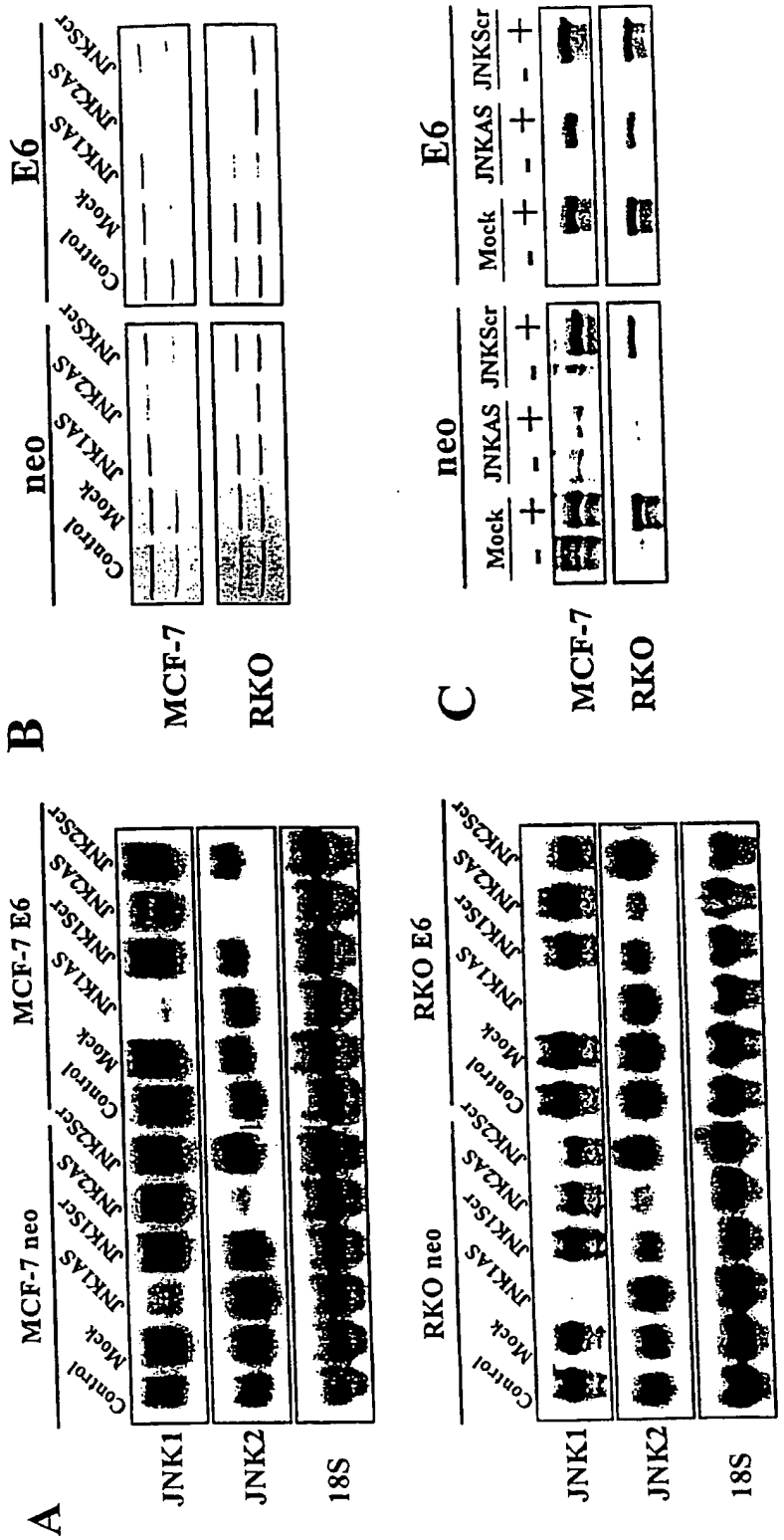

A
MCF-7 neo
MCF-7 E6
Control
Mock
JNK1AS
JNK1Scr
JNK2AS
JNK2Scr
JNK1
JNK2
18S
RKO neo
RKO E6
B
neo
E6
MCF-7
RKO
C
Mock
JNKAS
JNKScr
MCF-7
RKO

FIGURE 3

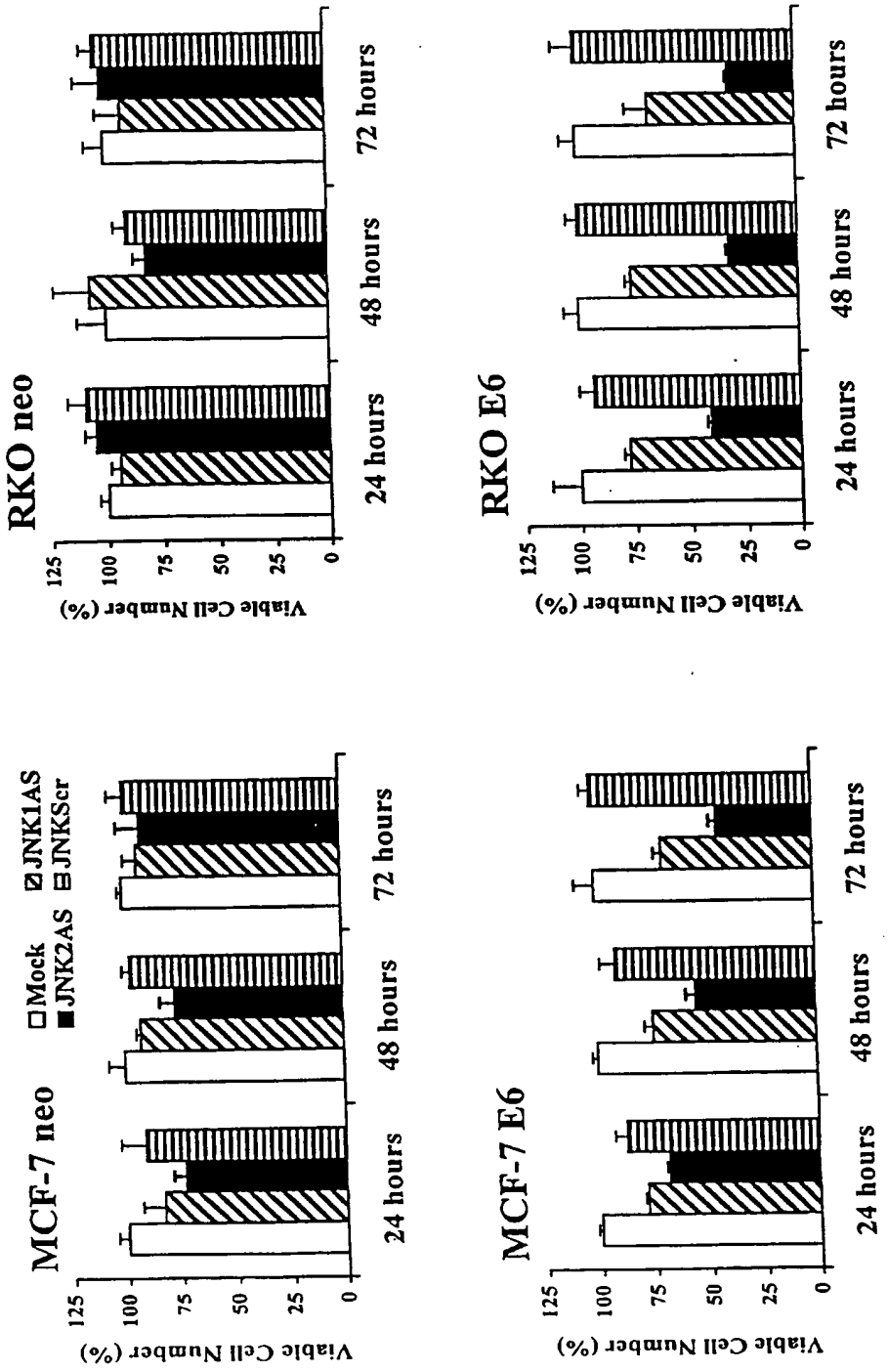

RKO neo
RKO E6
MCF-7 neo
MCF-7 E6
Viable Cell Number (%)
125
100
75
50
25
0
24 hours
48 hours
72 hours
Mock
JNK2AS
JNK1AS
JNKScr

FIGURE 4

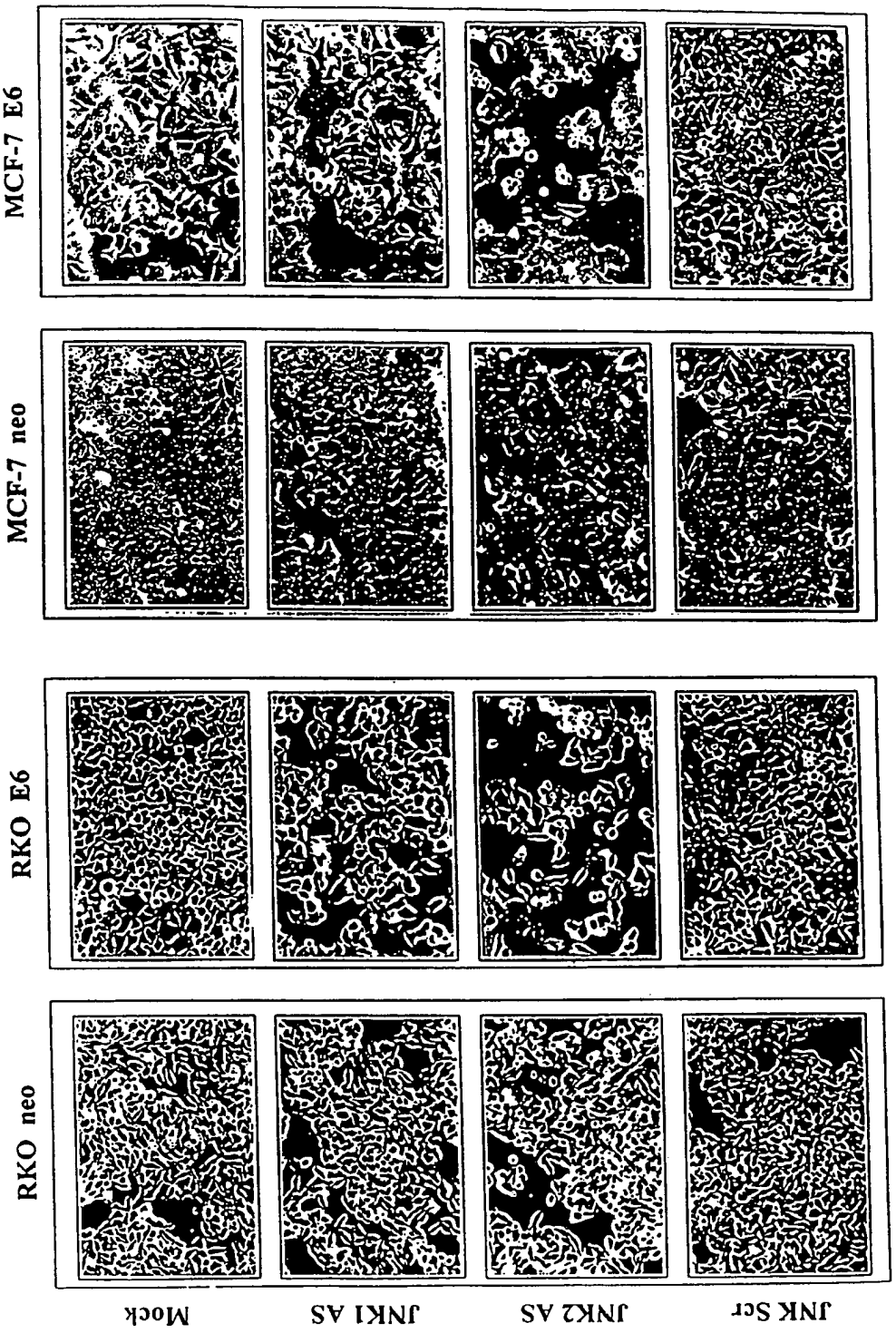


FIGURE 5

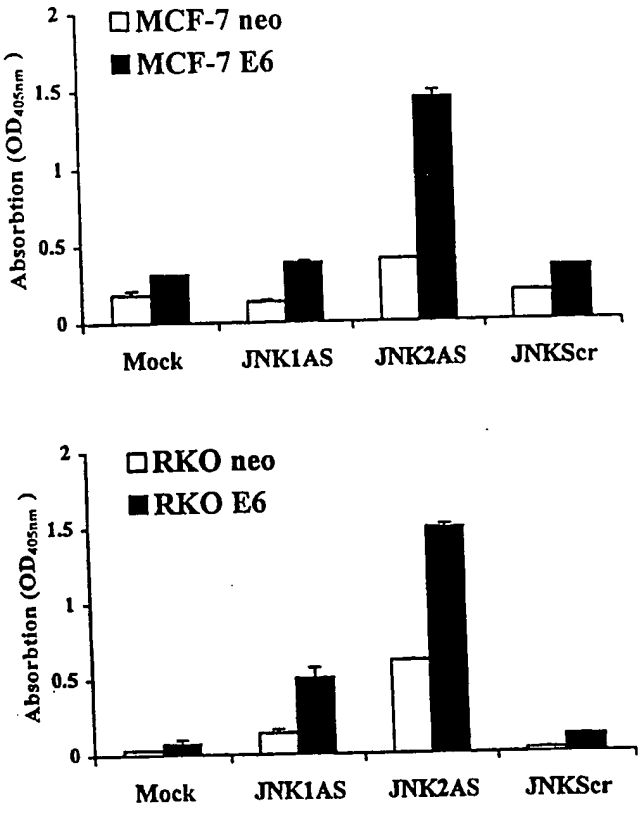

MCF-7 neo
MCF-7 E6
Absorbtion (OD405nm)
2
1.5
1
0.5
0
Mock
JNK1AS
JNK2AS
JNKScr
RKO neo
RKO E6
Absorbtion (OD405nm)
2
1.5
1
0.5
0
Mock
JNK1AS
JNK2AS
JNKScr

FIGURE 6

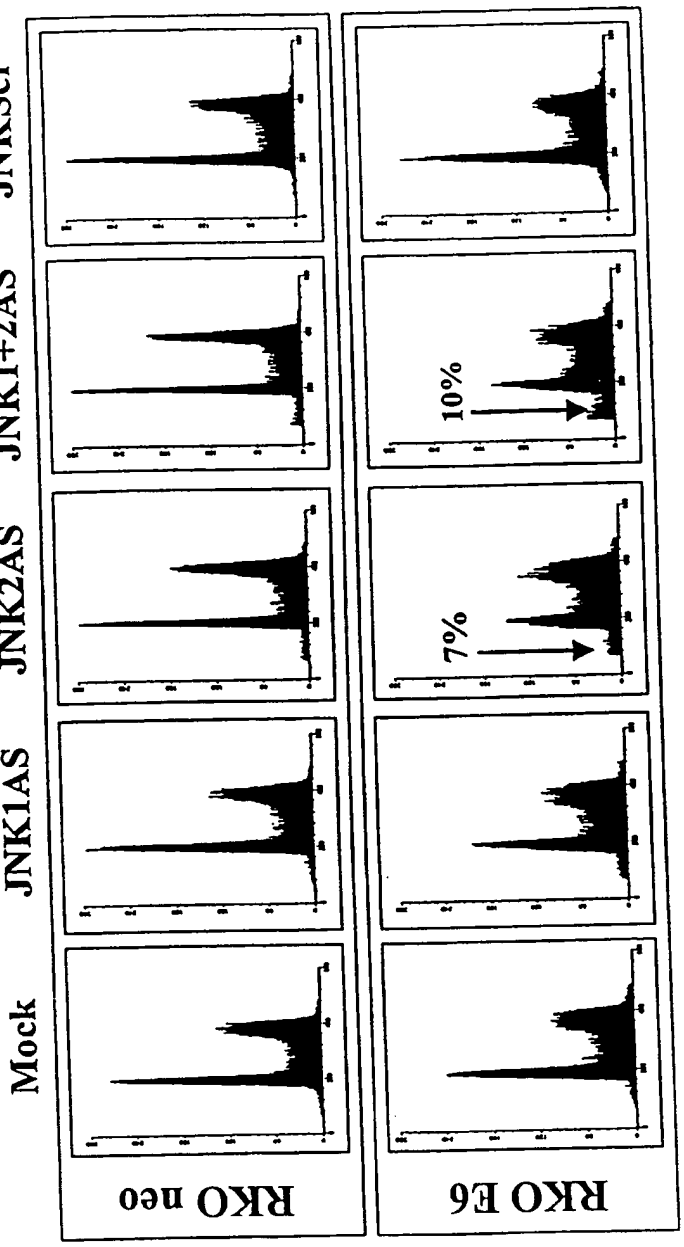


FIGURE 7

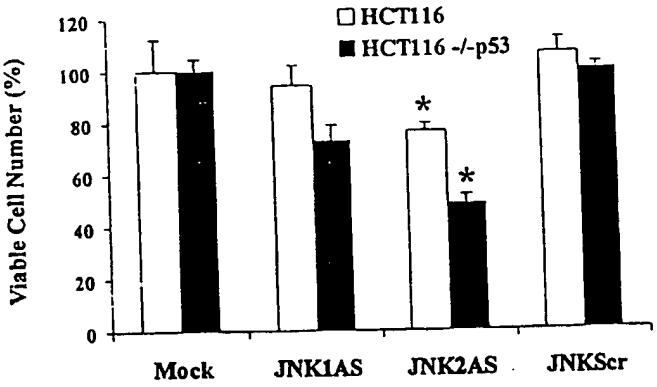
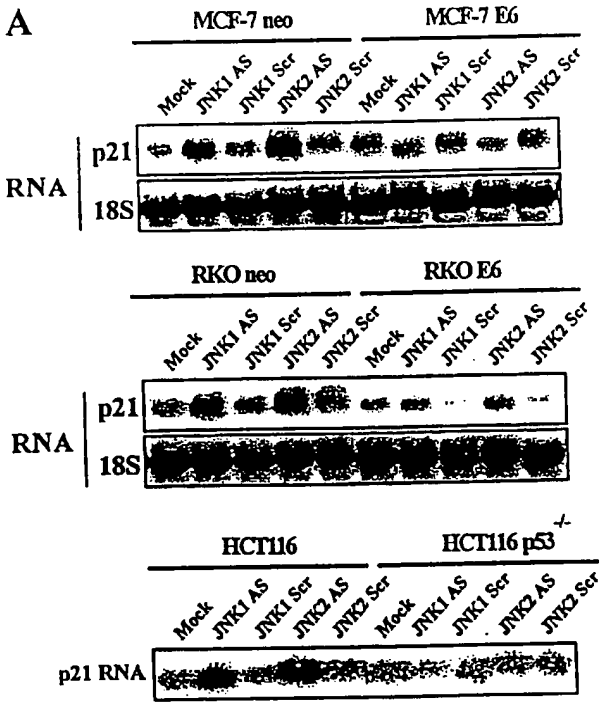

HCT116
HCT116 -/-p53
Viable Cell Number (%)
120
100
80
60
40
20
0
*
*
Mock
JNK1AS
JNK2AS
JNKScr

FIGURE 8


A
MCF-7 neo
MCF-7 E6
Mock
JNK1 AS
JNK1 Scr
JNK2 AS
JNK2 Scr
RNA
p21
18S
RKO neo
RKO E6
HCT116
HCT116 p53-/-
p21 RNA

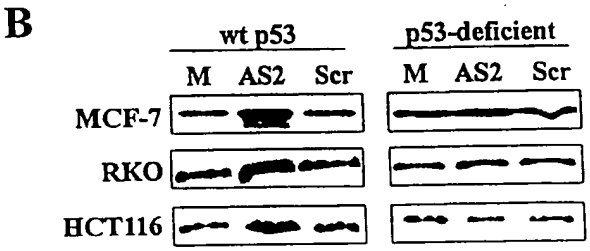

B
wt p53
p53-deficient
M
AS2
Scr
MCF-7
RKO
HCT116

FIGURE 9

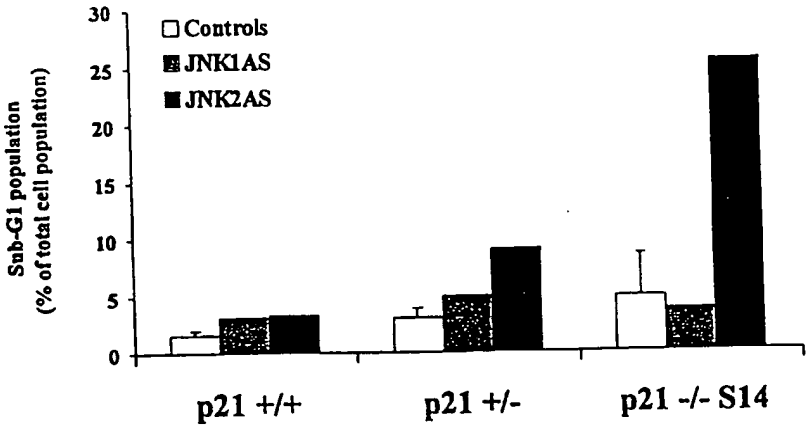

Sub-G1 population
(% of total cell population)
30
25
20
15
10
5
0
Controls
JNK1AS
JNK2AS
p21 +/+
p21 +/-
p21 -/- S14

FIGURE 10

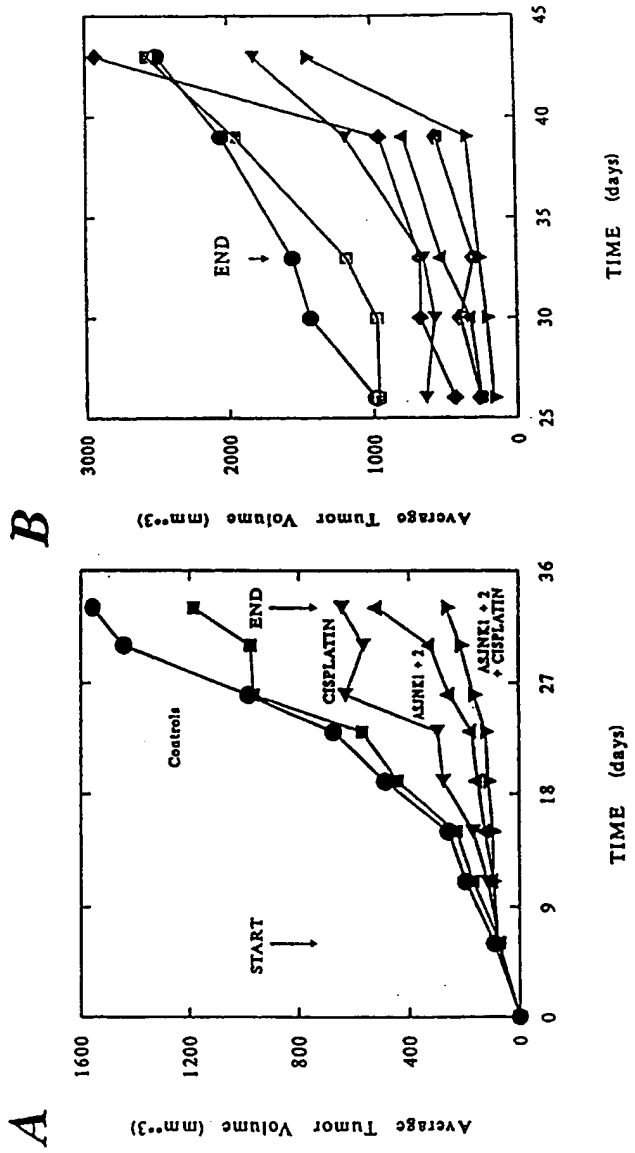

A
Average Tumor Volume (mm**3)
1600
1200
800
400
0
0
9
18
27
36
TIME (days)
START
END
Controls
CISPLATIN
ΔUNKI + 2
ΔUNKI + 2 + CISPLATIN
B
Average Tumor Volume (mm**3)
3000
2000
1000
0
25
30
35
40
45
TIME (days)
END

FIGURE 11

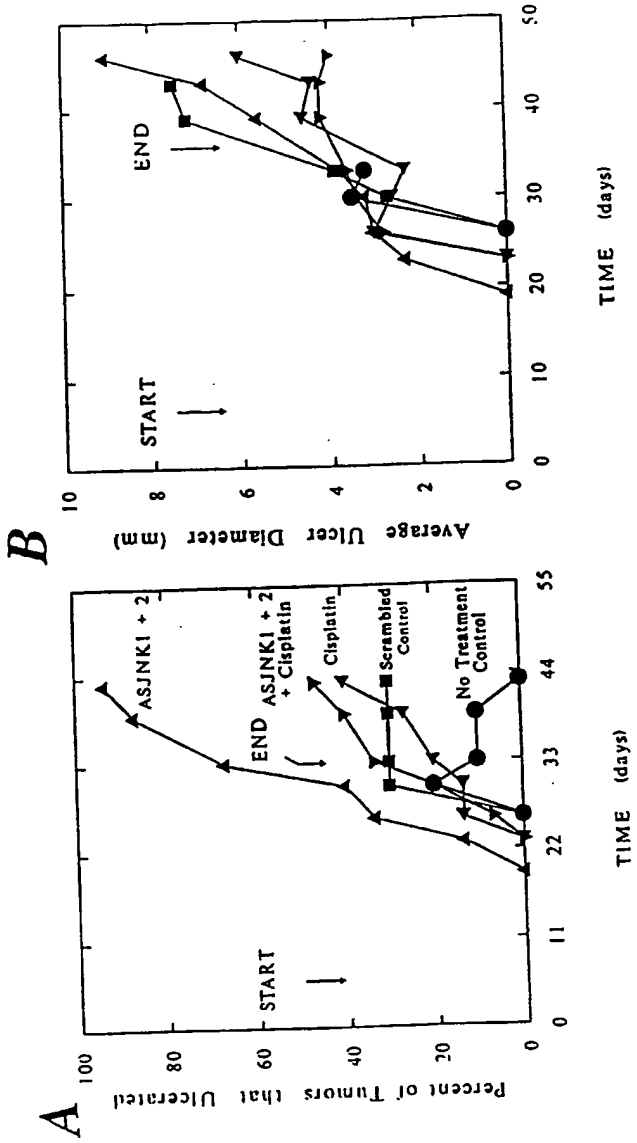

A
Percent of Tumors that Ulcerated
100
80
60
40
20
0
START
END
ASJNK1 + 2
ASJNK1 + 2 + Cisplatin
Cisplatin
Scrambled Control
No Treatment Control
0
11
22
33
44
55
TIME (days)
B
Average Ulcer Diameter (mm)
10
8
6
4
2
0
START
END
0
10
20
30
40
50
TIME (days)

FIGURE 12

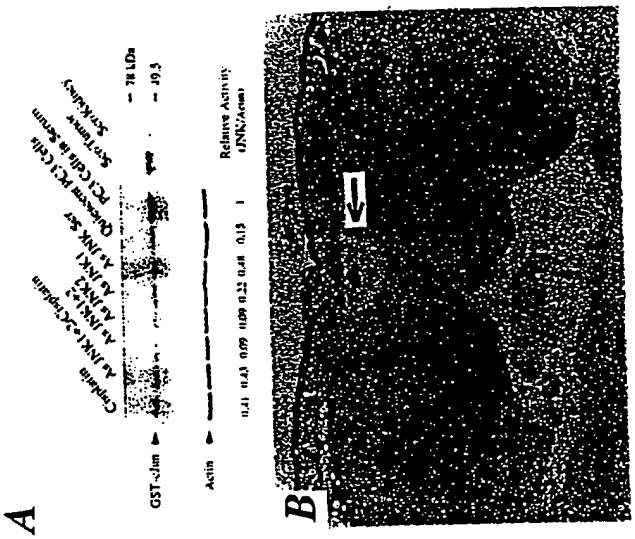
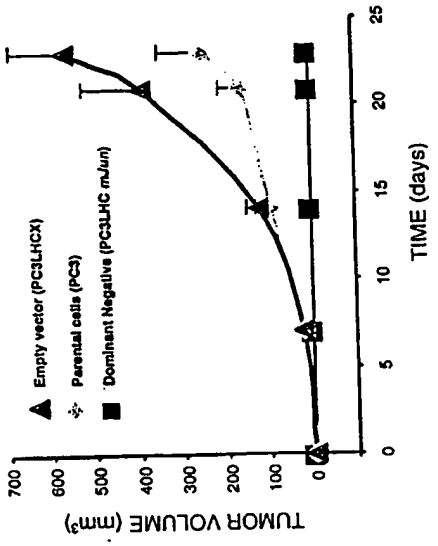

A
B
Actin
Relative Activity
(JNK/Actin)
0.41 0.43 0.09 0.09 0.22 0.48 0.13 1
GST-c-Jun
Cytochalasin
As-JNK1-cytochalasin
As-JNK1
As-JNK2
As-JNK1+2
As-JNK Scr
Quiescent PC1 Cells
PC1 Cells in Serum
Ser/Thr
Kinase
Activity
78 kDa
49.5

FIGURE 13


TUMOR VOLUME (mm³)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
TIME (days)
Empty vector (PC3LHCX)
Parental cells (PC3)
Dominant Negative (PC3LHC mJun)

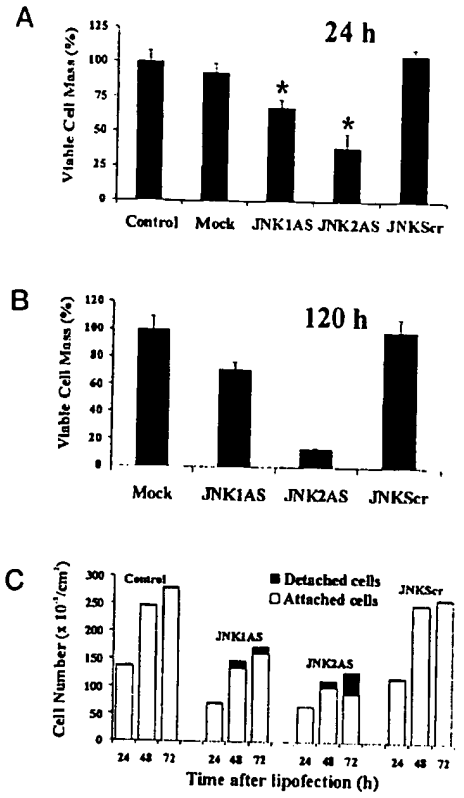

A
24 h
Viable Cell Mass (%)
125
100
75
50
25
0
*
*
Control
Mock
JNK1AS
JNK2AS
JNKScr
B
120 h
Viable Cell Mass (%)
120
100
80
60
40
20
0
Mock
JNK1AS
JNK2AS
JNKScr
C
Control
Detached cells
Attached cells
JNKScr
JNK1AS
JNK2AS
Cell Number (x 10⁻³/cm²)
300
250
200
150
100
50
0
24 48 72
24 48 72
24 48 72
24 48 72
Time after lipofection (h)

FIGURE 14

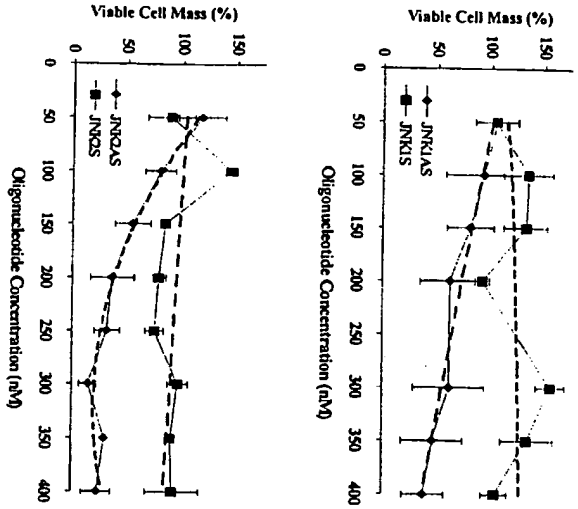


FIGURE 15

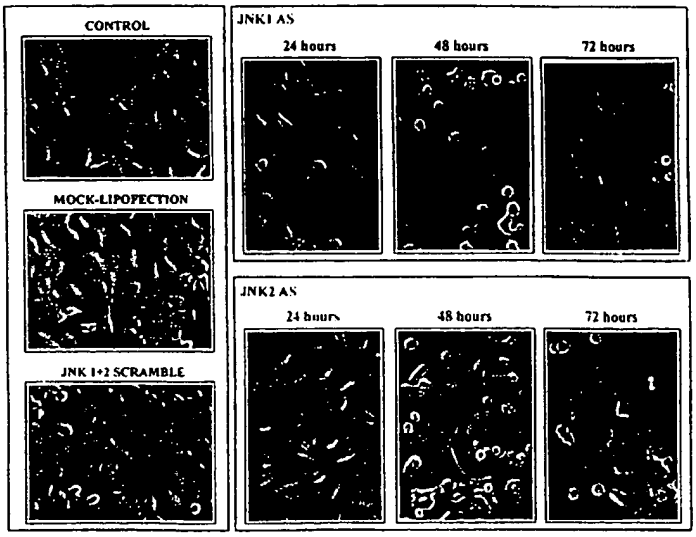


FIGURE 16

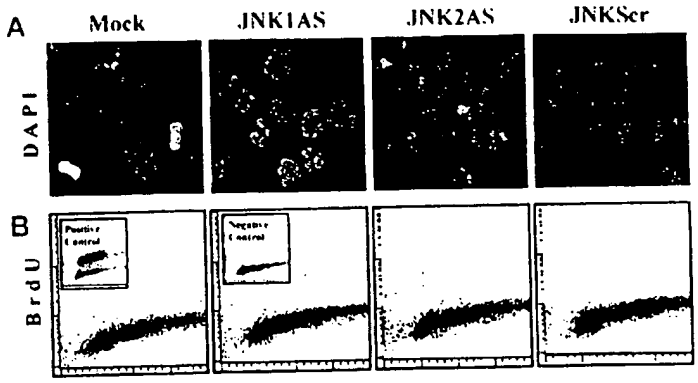


FIGURE 17

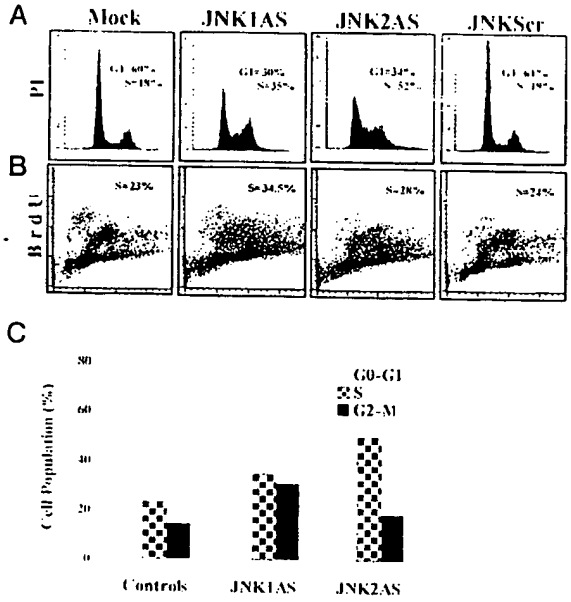


FIGURE 18

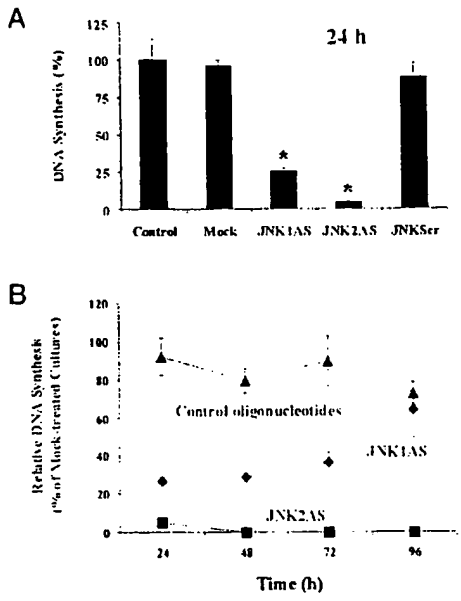


FIGURE 19

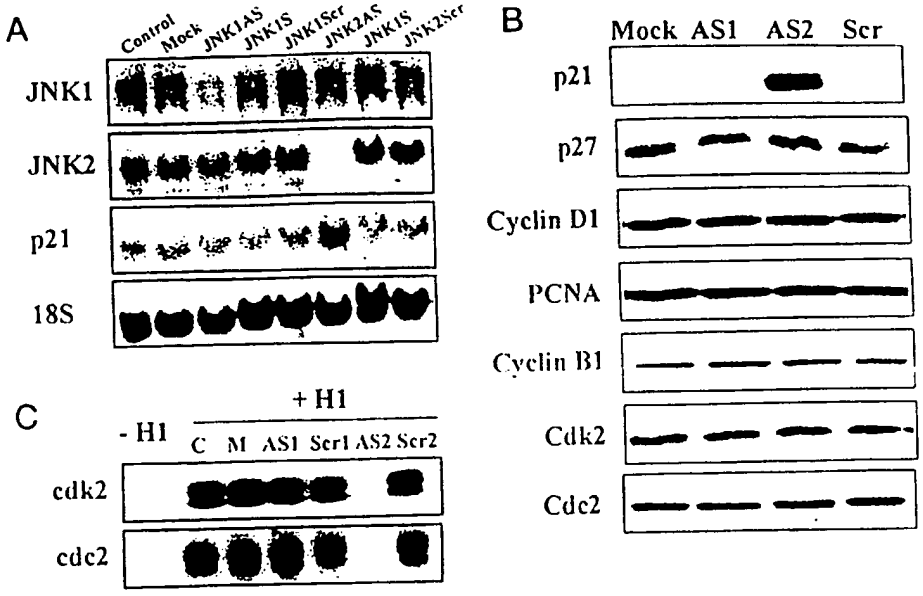


FIGURE 20

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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International Bureau

(43) International Publication Date
17 May 2001 (17.05.2001)

PCT

(10) International Publication Number
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(51) International Patent Classification[7]: C12N 15/11, A61K 47/48, 31/7088, C12N 5/10, C07H 21/00 // A61P 35/00

(21) International Application Number: PCT/US00/30869

(22) International Filing Date:
10 November 2000 (10.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/165,224 12 November 1999 (12.11.1999) US

(71) Applicant *(for all designated States except US)*: **THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **POTAPOVA, Olga** [RU/US]; 40 Fountain Ridge Circle, #2B, Baltimore, MD 21234 (US). **GOROSPE, Myriam** [ES/US]; 12707 Chesney Lane, Bowie, MD 20715 (US). **HOLBROOK, Nikki, J.** [US/US]; 3802 Timberview Way, Reistertown, MD 21436 (US).

(74) Agents: **MACKNIGHT, Kamrin, T.** et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
22 November 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/34792 A3

(54) Title: COMPOSITIONS AND METHODS FOR THE DIMINUTION OR ELIMINATION OF VARIOUS CANCERS

(57) Abstract: The present invention relates to the treatment of cancer utilizing Jun Kinase antisense (JNK2AS) mRNA. Additionally, the present invention relates to the screening of reagents that may augment or mimic the effect of JNK2AS treatment for cancer.

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/30869

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 A61K47/48 A61K31/7088 C12N5/10 C07H21/00 //A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 09214 A (NERO PAMELA SCOTT ;DEAN NICHOLAS (US); MONIA BRETT P (US); MCKAY R) 25 February 1999 (1999-02-25) page 6, line 27 -page 9, line 15 page 15, line 31 -page 21, line 15 page 50 page 84 -page 98; example 4 page 110; example 7	1-6,9-11
Y	SEQ IDs 16,28,31 and 43	1-13
Y	WO 94 23050 A (TARGETECH INC ;UNIV CONNECTICUT (US)) 13 October 1994 (1994-10-13) page 1, line 31 -page 2, line 23	1-6,9,10

-/--

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 June 2001	09/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FUCHS SERGE Y ET AL: "JNK targets p53 ubiquitination and degradation in nonstressed cells." GENES & DEVELOPMENT, vol. 12, no. 17, 1998, pages 2658-2663, XP002169469 ISSN: 0890-9369 cited in the application the whole document	6-8, 11-16
X	BOST FREDERIC ET AL: "The JUN kinase/stress-activated protein kinase pathway is required for epidermal growth factor stimulation of growth of human A549 lung carcinoma cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 52, 26 December 1997 (1997-12-26), pages 33422-33429, XP002169470 ISSN: 0021-9258 cited in the application	1-6,9-11
Y	the whole document	14-16
X	BOST FREDERIC ET AL: "The Jun kinase 2 isoform is preferentially required for epidermal growth factor-induced transformation of human A549 lung carcinoma cells." MOLECULAR AND CELLULAR BIOLOGY, vol. 19, no. 3, March 1999 (1999-03), pages 1938-1949, XP002169471 ISSN: 0270-7306 cited in the application the whole document	1-6,9-11
X	XU XIAOXING S ET AL: "A role for c-Raf kinase and Ha-Ras in cytokine-mediated induction of cell adhesion molecules." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 50, 11 December 1998 (1998-12-11), pages 33230-33238, XP002169472 ISSN: 0021-9258 cited in the application the whole document	1-5
A	FUCHS SERGE Y ET AL: "MEKK1/JNK signaling stabilizes and activates p53." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 95, no. 18, pages 10541-10546, XP002169473 Sept. 1, 1998 ISSN: 0027-8424 cited in the application	

-/--

2

INTERNATIONAL SEARCH REPORT

Inter *tonal Application No
PCI/US 00/30869

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SCHREIBER MARTIN ET AL: "Control of cell cycle progression by c-Jun is p53 dependent." GENES & DEVELOPMENT, vol. 13, no. 5, 1 March 1999 (1999-03-01), pages 607-619, XP002169474 ISSN: 0890-9369 cited in the application	
P,X	POTAPOVA OLGA ET AL: "Inhibition of c-Jun N-Terminal kinase 2 expression suppresses growth and induces apoptosis of human tumor cells in a p53-dependent manner." MOLECULAR AND CELLULAR BIOLOGY., vol. 20, no. 5, March 2000 (2000-03), pages 1713-1722, XP002169475 ISSN: 0270-7306 the whole document	1-13
P,X	POTAPOVA OLGA ET AL: "c-Jun N-terminal kinase is essential for growth of human T98G glioblastoma cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 32, 11 August 2000 (2000-08-11), pages 24767-24775, XP002169476 ISSN: 0021-9258 the whole document	1-6,9-11

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5 (all partially)

   A vector comprising a nucleic acid encoding SEQ ID 1, and cells containing it.

2. Claims: 1-5 (all partially) and 6-16 (totally)

   A vector comprising a nucleic acid encoding SEQ ID 2, cells containing it; its uses in methods for treating cancers or for screening compounds.

3. Claims: 1-5 (all partially)

   A vector comprising a nucleic acid encoding SEQ ID 3, and cells containing it.

4. Claims: 1-5 (all partially)

   A vector comprising a nucleic acid encoding SEQ ID 4, and cells containing it.

5. Claims: 1-5 (all partially)

   A vector comprising a nucleic acid encoding SEQ ID 5, and cells containing it.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 6-13 are directed to a method of treatment of the human/animal body, and claims 14-16 (as far as in vivo methods are concerned) are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte "ional Application No
PCI/US 00/30869

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9909214 A	25-02-1999	US 5877309 A	02-03-1999
		AU 730916 B	22-03-2001
		AU 8775098 A	08-03-1999
		EP 1003916 A	31-05-2000
		US 6221850 B	24-04-2001
		US 6133246 A	17-10-2000
WO 9423050 A	13-10-1994	AU 686614 B	12-02-1998
		AU 6497894 A	24-10-1994
		EP 0693130 A	24-01-1996
		JP 8508645 T	17-09-1996